The pH and Concentration of Hydrogen Peroxide – Impact upon Dental Enamel Properties and Response to Dietary Staining, Erosion, and Remineralisation

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Abstract

Hydrogen peroxide (HP) based whitening products can damage enamel due to their pH and concentration. This *in-vitro* study investigates the effect of HP pH and concentration on selected enamel properties. In addition, combined effects of whitening/remineralisation and subsequent resistance to simulated dietary erosion and staining were investigated.

Polished bovine enamel samples were treated with 6, 20, 40wt.% HP at pH 5, 7 and 9, for 2 hours daily for ten days. Samples were stored in artificial saliva at 37°C before and after treatment. Whitening/remineralisation investigations were performed using 6wt.% HP in combination with either casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) or nanohydroxyapatite (nHA). Samples were then subjected to simulated dietary staining using coffee and erosion using 0.3% citric acid (pH3.8). Measurements of enamel roughness (Ra), hardness (HV), and colour change (ΔE) were made before and after treatment using atomic force microscopy, micro-hardness testing, and spectrophotometry. Mineral loss and qualitative surface evaluations were undertaken using energy dispersive x-ray spectroscopy (EDX) and scanning electron microscopy (SEM), respectively, after treatment.

As HP concentration increased and pH decreased there was a statistically significant increase in ΔE ($P<0.05$). Greatest increase in Ra and decrease in HV occurred in enamel treated with pH9 40% HP. Remineralisation did not significantly affect ΔE, Ra, and HV of whitened enamel. CPP-ACP was effective in preventing statistically significant changes in Ra and HV values after erosive cycling. Enamel treated with nHA exhibited the greatest dietary stain uptake. No significant changes in enamel Ca, C, and P occurred after whitening, remineralisation, and erosion. SEM images revealed distinct surface changes mostly in pH9 and nHA treated samples.

All whitened specimens exhibited significantly greater colour change than the control group. Neutral HP caused the least damage to whitened enamel while producing a satisfactory whitening effect. Remineralising agents did not reduce whitening side effects, however, CPP-ACP prevented significant Ra and HV changes after dietary erosion.

To my family and friends,

Without whom none of my success would be possible

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List of main abbreviations

CP:	Carbamide peroxide
ANUG:	Acute necrotizing ulcerative gingivitis
HP:	Hydrogen peroxide
UV:	Ultraviolet light
ATSDR:	Agency for toxic substances and disease registry
CRCE:	Health England's centre for radiation, chemicals, and environmental hazards
GDC:	General Dental Council
ADA:	The American Dental Association
RDA:	Relative dentine abrasion procedure
SEM:	Scanning electron microscopy
PO:	Polyphenol peroxidase
CAT:	Catalase
SOD:	Superoxide dismutase
STPP:	Sodium tripolyphosphate
CPP-ACP:	Casein phosphopeptide-amorphous calcium phosphate
CPP-ACFP:	Casein phosphopeptide-amorphous calcium fluoride phosphate
CaSP :	Calcium sucrose phosphate
nHA:	Nano-hydroxyapatite
HAP:	Hydroxyapatite
Gly:	Glycine
Glu:	Glutamate
DMFT:	Decayed missing filled teeth
SEM:	Scanning electron microscopy
AFM:	Atomic force microscopy
EDX:	Energy dispersive x-ray spectroscopy
Ra:	Roughness average
HV:	Vickers hardness
ISO:	International organization for standardisation
ESEM:	Environmental scanning electronic microscope
LED:	Light-emitting diode
VAS:	Visual analogue scale
CIE:	The commission Internationale De L'Eclairage

WIC:	CIE whiteness index
WIO:	The optimized whiteness index
SBF-SEM:	Serial block-face imaging SEM
FIB-SEM:	Focused ion or plasma beam SEM
DW:	Distilled water
BL:	Baseline
AS:	Artificial saliva
BL:	Baseline
ΔE:	Overall colour change

Chapter 1. Introduction

Dental whitening is a commonly used minimally invasive treatment to improve smile aesthetics (Demarco *et al.*, 2009; Bezerra-Júnior *et al.*, 2016). Many reported *in-vitro* studies investigate the effects of commercially available whitening agents on enamel; mainly focusing on changes related to concentration differences between whitening products. There are few studies exploring the effects of whitening agent pH on enamel properties, and fewer studies addressing the combined impact of both pH and concentration of the whitening material on treated enamel surfaces. Studying the combined effects whitening agent pH and concentration have on enamel is necessary to gain a better understanding of advantages and side effects of a wide range of whitening products available in the market. An *in-vitro* study was set-up to investigate the combined effects different hydrogen peroxide (HP) concentrations and pH have on bovine enamel roughness, hardness, colour change, mineral composition, and surface quality.

Remineralising agents are commonly studied to assess their ability to restore enamel, previously subjected to various challenges, to its original state. Fewer attempts have been made to investigate their capacity to prevent expected surface damage as a result of such challenges. This study was designed to integrate two remineralising agents: casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and nanohydroxyapatite (nHA) into the whitening protocol in an attempt to assess their ability to prevent surface damage in whitened enamel and protect treated surfaces against dietary challenges thereafter.

In order to accomplish the best possible whitening outcome, the effects of dietary challenges on whitened enamel must be understood. Although *in-vitro* studies have investigated the staining and erosive effects of different foods commonly consumed by the public, there is a lack of understanding on how whitening agent concentration and pH, in addition to the application of a remineralising agent during whitening, impact the degree of surface damage and colour relapse as a result. This study was created to help understand the degree that dietary choices impact on whitened enamel by using commonly consumed beverages. This was done by exposing whitened and remineralised enamel to cyclic staining and erosion using coffee and citric acid which was set to simulate orange juice.

This novel study helps understand how whitening treatment options, i.e. whitening agent pH, whitening agent concentration, and the application of a remineralising agent affect the resultant whitening outcome both qualitatively and quantitatively, and the susceptibility of treated enamel surfaces to dietary erosion and staining.

Chapter 2. Literature Review

2.1 Tooth whitening agents

Aesthetic dentistry has evolved in relation to the high public demand in the 21st century. Public concern regarding smile aesthetics redirected clinicians and scientists towards formulating minimally invasive treatments such as tooth whitening, as an alternative to potentially destructive and harmful veneer or crown placements purely for cosmetic gain (Demarco *et al.*, 2009; Bezerra-Júnior *et al.*, 2016).

2.1.1 Historical background

In the late 1980s, whitening products (in-surgery and over the counter) were introduced to the U.S market, to accommodate for the high public demand in obtaining perfect white teeth (Carey, 2014). The whitening effect of carbamide peroxide (CP) on dentition was discovered during World War I, when it was used as an antiseptic agent to treat acute necrotizing ulcerative gingivitis (ANUG) (Banerjee and Millar, 2015). In 1962, Klusmier introduced the concept of using a CP-containing gel to treat inflamed periodontium after orthodontic treatment, which lead to the incidental discovery of the lightening effect of peroxide on enamel, and therefore, the possibility of using peroxides as tooth whitening agents. The personal communication sent by Klusmier to the Arkansas Dental Society was, however, left unnoticed until Haywood and Heymann described the technique in 1989 (Haywood and Drake, 1990; Haywood, 1991).

2.1.2 Chemistry of dental whitening

Carbamide peroxide is a stable structural complex that ultimately reacts with water and breaks down to its active components (Figure 1) (Price *et al.*, 2000). Its structural stability leads to its slow degradation, which allows for a prolonged active whitening process when compared to HP. HP is an unstable compound that decomposes into water and reactive oxygen radicals. It is highly soluble in water, giving an acidic solution with a pH that differs according to the concentration, for example a 1% HP solution was reported to have a pH of between 5 and 6 (Walsh, 2000).

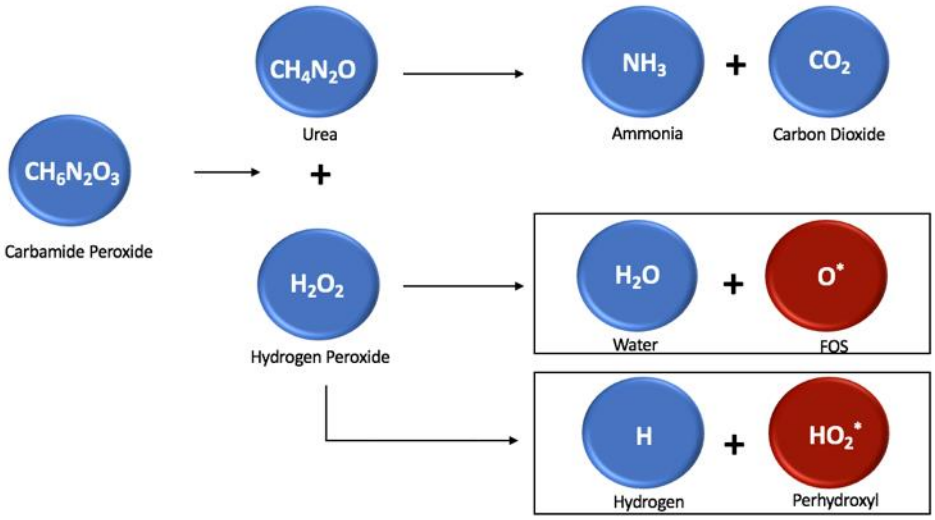


Figure 1 Shows the breakdown of CP to hydrogen peroxide and urea. Hydrogen peroxide will then breakdown into water and free oxygen species (FOS) which actively degrades chromogens. At higher pH levels >7 there is a greater chance that HP will breakdown forming hydrogen and perhydroxyl which leads to better whitening results as compared to FOS.

Tooth whitening products may contain HP as an active agent, glycerine as a carrier, carbopol as a thickening agent, and finally, a number of flavouring agents (ADA, 2008; Thickett and Cobourne, 2009). Whitening occurs through the process of chemical degradation of chromogens. Chromogens are the cause of dental discolouration, and are either present as large organic compounds with double bonds, or as metallic containing compounds, the latter being less likely to be whitened using HP. In contrast, oxygen radicals released by HP react with organic chromogens more effectively through an oxidising process which breaks the strong double bonds, destabilising the chromogenic compound, and ultimately reducing tooth discolouration (Carey, 2014).

Whitening agents can be either products that can be applied with no activation step, such as HP on its own, or that require activation by either light, chemical mixing, or a combination of both. Controversy over the most efficient and least harmful activation system is evident in the literature (Baroudi and Hassan, 2014). Chemical activators such as manganese gluconate, manganese chloride, and ferrous sulphate have been reported to improve the performance of whitening gels through accelerating the chemical reaction of HP on the enamel surface, therefore reducing exposure time and post-whitening sensitivity (Buchalla and Attin, 2007; Batista *et al.*, 2011). Ziemba *et al*, on the other hand, claim that the addition of a photo Fenton activator to the whitening gel and using an ultraviolet light activation system (UV) for 45 minutes significantly improves the Vita® shade score as compared to a chemically activated system (Ziemba *et al.*, 2004). Other studies disagree with this, claiming no spectrophotometric shade difference after using a light activation source such as ultraviolet light or plasma arc light (Polydorou *et al.*, 2008; Baroudi and Hassan, 2014). It is important to note that in the study by Polydorou *et al*., whitening was conducted for short time intervals averaging 18 minutes, which might not be enough to produce any significant findings.

Some argue that short in-surgery light activation treatment leads to the absorption of energy which transforms into heat, causing pulpal damage and enamel dehydration, the latter giving the illusion of whiteness (Polydorou *et al.*, 2008; Mollica *et al.*, 2010). The exact whitening process as explained in the literature is vague and many whitening studies do not allow for the full remineralisation cycle by saliva to take place where many side effects subside. There seems to be a consensus, however, on the oxidation process of chromogens by active whitening ingredients, but opinions diverge in regards to the ideal whitening activation process (Majeed *et al.*, 2015b).

2.1.3 Toxicity of whitening agents

HP is a powerful oxidizing agent that has the potential to causes irritation to the skin, eyes, and mucous membranes upon exposure to high concentrations (>10wt.%) (ATSDR, 2014). It generates hydroxyl radicals, which cause lipid peroxidation, DNA damage, and cellular death (CRCE, 2009). The maximum reported concentration of HP without causing mucosal irritation was 5% with damaging effects seen at 8%. Much higher concentrations can be formulated and HP is considered a corrosive substance at 50%. Toxicity of whitening agents are dependent on the concentration of HP, composition of the whitening agent, and duration of treatment (Carey, 2014). Upon application, the low molecular weight of HP enables it to penetrate to the dental pulp chamber and periodontal ligament, causing an inflammatory reaction that might be sufficient to initiate cervical root resorption, and damage to pulp, fibroblasts, or DNA (Bahuguna, 2013; Fernandes *et al.*, 2013). Reactive oxygen radicals may cause genotoxicity and cytotoxicity, but unless administered in very high concentrations (30% HP), these radicals are reported to be unable to cross cell membranes and inflict damage (Tredwin *et al.*, 2006).

Guidelines were developed and policies put in place to regulate the use of HP, and ensure public safety and wellbeing. The Cosmetic Products Enforcement Regulations in the UK set the maximum allowed concentration of HP to be used for dental whitening to 6% (GDC, 2011; CPER, 2013), and according to the national guidelines from WorkSafe in Australia, HP is considered a hazardous substance in concentrations above 5% (Walsh, 2000). The American Dental Association (ADA) awards the seal of acceptance to products having the maximum concentration of 10% CP (3.5% HP) (ADA, 2008). Reported concentrations of commercially available products, however, ranged up to 40% according to previously published studies (Kwon *et al.*, 2013; Lubbadeh *et al.*, 2018). To date, long term adverse effects caused by whitening agents used in accordance with guidelines and regulations have not been reported (Hasson *et al.*, 2006; ADA, 2008; Carey, 2014; Soares *et al.*, 2015). Longitudinal evaluation of the long term adverse effects of whitening agents is required, including potential impact on the overall structural and mechanical integrity of tooth structure.

2.1.4 Vital tooth whitening techniques

In-surgery whitening techniques

In accordance with UK regulations (GDC, 2011), in surgery whitening may only be performed by a registered dental professional, dental hygienist, dental therapist, or a clinical dental technician under the direct supervision and direction of a dentist, and cannot be carried out for example by beauty therapists. Historically, a whitening gel with high concentrations of chemically or light activated HP is applied for a short duration (45min-1hr). The high concentration of HP usually used for in-surgery products (30-38%) requires less time to release high levels of free oxygen radicals, thus, immediate results are observed following the whitening procedure (Hafez *et al.*, 2010; Banerjee and Millar, 2015). According to the literature, it is possible to get the tooth colour 5-8 shades lighter following multiple whitening cycles, with stability in results for up to 9 to 24 months (Tay *et al.*, 2012; Cartagena *et al.*, 2015). Although, surgery whitening products attracted attention for their ability to immediately whiten teeth, a high association with post-treatment tooth sensitivity and soft tissue ulcerations was noted (Tano *et al.*, 2012; Dias *et al.*, 2015).

In an effort to minimise sensitivity while maintaining whitening efficacy, the incorporation of a titanium dioxide photo-catalyst into lower concentrations of HP (3.5-6%) was proposed. Exposure to light of a wave length ranging from 380-450nm accelerates the HP reaction and release of radicals, improving enamel colour (Suemori *et al.*, 2008; Tano *et al.*, 2012). This novel approach is as effective in whitening with less post-whitening sensitivity when compared to a 35% HP whitening agent (Suemori *et al.*, 2008; Bortolatto *et al.*, 2014). Skocaj *et al*, however, argue that titanium dioxide has the disadvantage of inducing oxidative stresses which ultimately cause cell damage, inflammation, and genotoxic side effects (Skocaj *et al.*, 2011). While the results published to date on titanium dioxide containing whitening agents are promising, long term effects and outcome stability are yet to be investigated and therefore, results must be viewed with some caution.

Dentist supervised at-home whitening techniques

Night-guard vital whitening is considered the gold standard in tooth whitening and commonly prescribed by dentists. It is self-administered by patients, with fewer reported side effects, and is a more cost effective whitening solution (Alqahtani, 2014). This whitening process includes the application of 10% CP to a custom tray and worn overnight for 2-6 weeks (Haywood, 1997). Evidence suggest that At-Home whitening using 10% CP applied for 8-10 hours, nightly, for 14 days has more than double the overall whitening effect (ΔE) (ΔE= 12.3) of a 35% HP gel applied In-Surgery for 30 minutes once a week for two weeks (ΔE=5.3) (Zekonis *et al.*, 2003). These findings were confirmed by another clinical study (Matis *et al.*, 1998). A subsequent clinical study did not find this (Basting *et al.*, 2012), a 10% CP gel applied for 2 hours, nightly, for 3 weeks showed no significant difference in its whitening effect in comparison to a 35% HP gel applied for three 8 minute cycles, once a week, for a total of three weeks. Both techniques showed a median shade change from baseline (BL) of 4-7 Vita®-shade guide units. The subjective evaluation of colour change in this study may have led to a greater margin of error in comparison to the objective readings obtained in the studies using a colorimeter (Matis *et al.*, 1998; Matis *et al.*, 2000; Zekonis *et al.*, 2003). Considering differences in shade measurement techniques, results indicate that At-Home night guard dental whitening using 10% CP produces similar or a greater whitening effect in comparison to In-Surgery whitening.

At-home day whitening techniques range from 2-4 hours daily for an average duration of 2 weeks. The reported ΔE after treatment using a 15% CP gel applied for 2 hours, daily, for 2 weeks range from 4.6-5.3 (Matis *et al.*, 2002), lower than that reported for the night guard dental whitening using a lower CP concentration. This is attributed to the difference in the whitening duration; 2-4 hours during the day and 8-10 hours during the night. The limited exposure time of CP is important to consider, as only 50% of CP breaks down to its active components after 2 hours of treatment (Nathoo *et al.*, 1996; Matis *et al.*, 1999).

Over the counter whitening techniques

Over the counter (OTC) dental whitening products are purchased and applied without professional supervision. The whitening gel is usually carried in disposable plastic trays, on plastic strips designed to fit labial and buccal surfaces of teeth, carried in containers to be applied using a brush, or incorporated in tooth pastes and mouth rinses. Active whitening agents commonly present in OTC products are CP or HP, however, legal restrictions on HP and CP based products enforced by the EU Council Directive 2011/84/EU, have led to the rescission and discontinuation of their production in many markets across Europe. The directive states that '*Tooth whitening or bleaching products containing concentrations greater than 0.1 % or less than 6 % of H_2O_2 (hydrogen peroxide), present or released are to be only sold to dental practitioners*.' (Union, 2011); making the use of products containing less than 0.1% HP insufficient to achieve the desired whitening results. In an attempt to overcome this issue, new OTC whitening products were introduced into the market with new active ingredients such as sodium chlorite, sodium carbonate peroxide, and phthalimidoperoxycaproic acid (PAP).

According to an *In-vitro* study on human enamel, commonly purchased OTC whitening products containing sodium chlorite or PAP showed the greatest enamel structural alteration according to scanning electron microscopy; visible as etching patterns (Greenwall-Cohen *et al.*, 2019). In addition, there was a significant reduction in enamel hardness (Vickers) compared to other products. Results also showed that sodium carbonate peroxide and PAP based products, surprisingly, produced less colour change than saline which was used as a negative control. Sodium chlorite, on the other hand, produced a greater whitening effect than CP which was used as a positive control. Sodium chlorite additionally caused a greater reduction in enamel hardness in comparison to HP and CP (Zantner *et al.*, 2007). This could be attributed to the presence of citric acid in combination with sodium chlorite in many OTC whitening products which could result in the structural alterations and reduction in hardness due to their low pH value.

2.1.5 Whitening toothpastes

Whitening toothpastes are popular products; with high public demand. The efficacy of removing extrinsic stains is determined by the physical characteristics of minerals within whitening toothpastes (de Melo Monteiro *et al.*, 2016). Whitening toothpastes act by chemically whitening and/or abrasively removing extrinsic stains (Joiner, 2006). Aside from the standard constituents of toothpastes such as fluoride, the active whitening agents include HP, CP, or sodium citrate, which chemically whiten enamel, and silica, calcium carbonate, or alumina to abrasively remove extrinsic stains (Joiner, 2010).

Other factors to consider in addition to the composition of whitening toothpastes, include particle size of abrasives incorporated and the type of tooth brush used. Measurements obtained by a surface profilometer, reveal that larger abrasive particles cause more enamel damage than smaller ones, and a medium textured tooth brush proved to be 1.4 times more abrasive than a soft tooth brush (De Boer *et al.*, 1985).

This was not the case, however, in another laboratory study where a soft toothbrush caused higher levels of surface loss than a medium and a hard toothbrush (Dyer *et al.*, 2000). This was attributed to their tendency to flex more easily than the medium or hard bristles, therefore carrying larger quantities of abrasive paste particles to areas inaccessible by harder bristles.

The role of tooth brush filaments in abrading the tooth surface has been supported by Lewis *et al.*, however, they claim that there is little evidence determining the degree abrasive particles impact the enamel surface, as results vary in accordance to the brushing motion, concentration, and size of particles (Lewis *et al.*, 2004).

Although some authors report significant improvement in tooth colour following the use of HP containing toothpastes (Kleber *et al.*, 1998) or a combination of HP and hydrated silica whitening toothpastes (Kakar *et al.*, 2004), the effectiveness of one or both modes of action (chemical and mechanical) have been called into question (Schemehorn *et al.*, 2011; Soares *et al.*, 2014a). According to results gathered using the radioactive Relative Dentine Abrasion procedure (RDA), used to quantify the amount of abraded surface, it has been concluded that whitening toothpastes were more abrasive than non-whitening toothpastes (Schemehorn *et al.*, 2011). In addition, chemicals added to whitening toothpastes such as HP have no significant whitening effect due to deeply seated stains and/or to the short contact time, making the very low HP percentage commonly used in toothpastes (1%) inefficient (Soares *et al.*, 2014a).

2.1.6 Developments in whitening agents

Plant extract

In an effort to effectively replace commercially available whitening agents with natural, less cytotoxic whitening products, a whitening gel containing fruit organic acids (oxalic, citric, tartaric, malic, succinic, and fumaric) with pH values ranging from 4.5 to 7, was compared to CP gel. Organic acids were extracted and mixed with 15% hydroxyapatite and zinc-hydroxyapatite, then added to human fibroblasts in addition to 60 resin composite samples for 8 hours/day for 5 days. Following exposure to both whitening protocols, fibroblasts were tested for toxic side effects and resin composites for colour changes. Similar levels of whitening were achieved however, fruit organic acids were less cytotoxic than HP (Baldea *et al.*, 2016). Although the aforementioned study tested colour changes on resin composites, it has been reported in the literature that the whitening effect recorded in enamel specimens exposed to fruit acids is attributed to the erosive effect fruit acids have on enamel which additionally causes a significant reduction in Ca and P ions (Lee *et al.*, 2012; Lee and Bae, 2016).

Naturally based whitening toothpastes were reported to be more effective than synthetic toothpastes in removing extrinsic enamel stains (Kalyana *et al.*, 2011). Using digitally analysed images by Adobe® Photoshop software, results showed that brushing 24 human enamel specimens using an electric toothbrush and a toothpaste containing Papain, Bromelain, Miswak, Neem and 1000ppm fluoride as novel active ingredients, produced a greater whitening effect when compared to the control group (calcium carbonate, sorbitol, titanium dioxide, sodium silicate, sodium saccharin and 1000ppm fluoride).

The addition of vegetable-derived enzymes as a means for enhancing whitening efficacy and reducing enamel structural changes post-whitening was reported in the literature (Chakravarthy and Acharya, 2012; Gopinath *et al.*, 2013). Sweet potato enzymes including polyphenol peroxidase (PO), catalase (CAT), and superoxide dismutase (SOD) were added to two concentrations of HP (10% and 35%) and tested on 32 artificially stained teeth (Gopinath *et al.*, 2013). Sweet potato extract contains a number of antioxidant molecules, which according to studies, are highly effective free radical scavengers that target chromogens (Heim *et al.*, 2002; Teow *et al.*, 2007; Rautenbach *et al.*, 2010). In comparison to HP without the additive antioxidants, spectrophotometric and SEM images of the experimental group revealed significantly increased tooth whiteness levels and reduced enamel structural breakdown (Gopinath *et al.*, 2013). The added antioxidant to HP reduced its high activation energy and increased the rate of free radical release. Lowering the activation energy increases the rate of free radical release, producing the desired whitening effect in less contact time, and ultimately

causing less damage to enamel microstructure (Gopinath *et al.*, 2013). Although, the aforementioned *in-vitro* studies showed improved results that could potentially advance the field of dental whitening, these techniques have not been clinically tested, and therefore results can only be viewed as potentially promising.

Chemical additives

There are many novel dental whitening products with chemical additives (Hyland *et al.*, 2015), bioactive additives (Al Batayneh, 2009; Farooq *et al.*, 2013; Somasundaram *et al.*, 2013), and natural organic additives (Kalyana *et al.*, 2011; Baldea *et al.*, 2016). In an effort to produce greater whitening results using lower concentrations of whitening agents, researchers formulated a new complex. This complex was composed of 5% HP, sodium tripolyphosphate (STPP), and urea, and was compared to a whitening agent containing 10% HP and urea (Hyland *et al.*, 2015). This randomised double-blind clinical trial revealed that lower concentrations of HP in the (HP (5%) + STPP + urea) complex were as effective in whitening according to spectrometer measurements as a (10%HP + urea) complex. This highlights the potential to maximise whitening efficiency using lower concentrations of HP in the presence of chemical additives, which would help clinicians and patients achieve desired whitening results mostly obtained through the application of higher concentrations of HP.

The incorporation of calcium peroxide nano-particles allows active whitening ingredients to deeply penetrate enamel micro- and nano-structures, resulting in an increased surface contact and ultimately a greater whitening effect (AlKahtani, 2018). Another development was the incorporation of calcium phosphate microspheres as CP carriers in an attempt to reduce structural damage and tooth sensitivity (Mellgren *et al.*, 2018). The carrier system proved to be promising, not affecting diffusion rates of CP through enamel. The introduction of these new additives and carrier systems can potentially maximise the whitening effect and reduce or even eliminate any potential side effects.

2.2 Effect of hydrogen peroxide pH on enamel

As more whitening products are introduced, focus in research has been mostly on the overall impact of whitening agents on enamel surface morphology and mechanical behaviour, and not on the level of contribution peroxide pH has on the initiation of a destructive chain reaction during the whitening process. According to laboratory studies, enamel exposed to whitening products with different pH levels showed an increased risk of enamel demineralisation and root resorption upon prolonged exposures to highly acidic products with pH values falling below 5.2 or highly basic products >7 (Driessens *et al.*, 1986; Price *et al.*, 2000).

The pH values of 26 commercially available tooth whitening products in Canada, ranged from 3.67 indicating a highly acidic product, up to a highly basic product with a pH value of 11.13 (Price *et al.*, 2000). Twenty-one commercially available tooth whitening products in the South African market had similar pH values, reporting a minimum pH of 3.76 and a maximum pH value of 9.68 (Majeed *et al.*, 2011a). Products in Brazil and Iran had reported pH values as low as 2.39 and 2.97 respectively (Freire *et al.*, 2009; Jamshidian *et al.*, 2016). Whilst the erosive effects acids inflict on enamel have been widely studied, exposing enamel to alkaline agents proved to be equally destructive. Alkaline products breakdown organic matter, such as proteins, which is the main constituent of the protective pellicle surrounding enamel surfaces. Such products also target proteins present in the enamel microstructure, mainly amelogenin, which encapsulates enamel prisms, connects prisms to each other, and links mineral crystals within each prism (Taube *et al.*, 2010). The loss of organic matter in enamel by alkaline products makes it vulnerable to subsequent acidic attacks, due to the removal of numerous organic barriers that normally prevent or delay exposure of the apatite crystals.

The pursuit for an optimal whitening agent pH have lead scientists to formulate a neutral HP by either adding sodium hydroxide or hydroxyapatite. It proved to be significantly less destructive to enamel when compared to acidic HP. This was attributed to the alkaline salt evenly adhering to enamel surface lessening the direct contact between HP and enamel, thus, forming a protective layer (Sun *et al.*, 2011). Superiority of alkaline and neutral whitening agents was evident in a laboratory study which subjected human enamel to acidic, neutral, and alkaline 30% HP solutions. Colour values revealed a greater whitening effect with least structural change in enamel whitened using neutral or alkaline HP compared to the acidic whitening agent (Xu *et al.*, 2011). The lack of any structural changes in enamel whitened with alkaline or neutral HP was attributed to the oxidation reaction occurring in the dentine instead of enamel, therefore resulting in superior whitening with little or no harm to enamel. The erosive damage of the acidic HP on the other hand, was confined to the external surface of enamel, not penetrating deep into enamel and dentine which could explain the reported limited

whitening effect. Consensus on the superiority of Alkaline HP, however, did not exist in the literature. Alkaline HP alters enamel morphology through accelerating the oxidation reduction reaction (Araujo *et al.*, 2013). This could be attributed to the greater HP: caustic soda ratio required for the creation of an alkaline solution, causing an auto-accelerating reaction that generates oxygen and heat; leading to irreversible damage to enamel (Hart *et al.*, 2013; Jurema *et al.*, 2018).

The fluctuating and inconsistent findings when studying the effects of HP pH is attributed to the varying components of the whitening gel used as opposed to being purely caused by their pH value. Research suggests that at a constant pH value of 7, whitening efficiency of HP is influenced by the type of conditioner added (Ito *et al.*, 2019). Adding pH conditioners such as sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), and potassium bicarbonate ($KHCO_3$) to HP revealed that in the cations, the potassium ion had a greater whitening effect than the sodium ion, and the bicarbonate ion had a greater whitening effect than the hydroxide and carbonate ions in the anions. Therefore, the greatest whitening effect of HP with controlled concentration and pH was recorded when $KHCO_3$ was added. The least whitening effect under the same circumstances was recorded in the K_2CO_3 and Na_2CO_3 groups (Basting *et al.*, 2005; Araujo *et al.*, 2013).

2.3 Potential side effects of whitening

2.3.1 Changes in Enamel Hardness

Tooth whitening agents cause a number of adverse effects on the hardness of human enamel, making it more susceptible to deformation and fracture (Akal *et al.*, 2001; Araujo *et al.*, 2013; Lia Mondelli *et al.*, 2015). Enamel specimens exposed to 7.5% HP exhibit a significant reduction in enamel hardness (Knoop hardness reduced by more than two thirds, from 294 to 78 MPa) (Pinto *et al.*, 2004). This is explained by the oxidation process that the organic and inorganic components of enamel undergo when exposed to whitening agents. This leads to changes in enamel morphology by the development of porosities and micro-cracks, ultimately causing a reduction in hardness (Markovic *et al.*, 2007; Eva *et al.*, 2013).

Furthermore, the pH of whitening products plays an important role in determining the degree of impact whitening agents have on the hardness of whitened enamel. Whitening agents with acidic pH cause greater reductions in hardness when compared to products with neutral or slightly alkaline pH (Furlan *et al.*, 2017). It has been reported that whitening using a 25% HP with a pH of 3.2 cause a significantly greater decrease in enamel hardness in comparison to a 38% HP whitening product with a pH of 6.7 (Eva *et al.*, 2013). In contrast, research has also revealed that whitening using two 10% CP products with pHs 6.79 and 6.23 for 8hrs/day for a duration of 14 days have resulted in no significant changes in enamel hardness (Rodrigues *et al.*, 2001; Leonard *et al.*, 2005). All studies reported had artificial saliva as a storage medium for whitened enamel to provide samples with an opportunity to undergo remineralisation between whitening cycles.

According to the literature, enamel hardness changes occurring post whitening are irrespective of the type of light activation source used. Araujo *et al.*, claimed that enamel hardness reduction recorded post whitening was 5.81% regardless of the type of light used (LED, Halogen, or Argon Laser) (Araujo *et al.*, 2010). This indicates that enamel mechanical changes post-whitening appear to be dependent on the chemical reaction and oxidation process, which, in turn, is directly proportional to the concentration and pH of whitening agents used, and is independent of the type of activation source used to initiate the reaction (Alqahtani, 2014).

Additionally, the duration of application was reported to have a greater impact on enamel micro-hardness values post whitening than HP pH and concentration (Jurema *et al.*, 2018). Limited exposure time could possibly contribute to the absence of any significant side effects in whitened enamel, and this could mean that the impact of pH and concentration become significant when the exposure time exceeds a certain limit.

Other whitening gel components have been reported to cause a significant reduction in micro-hardness values. Enamel treated using a product containing carbopol caused a significant micro-hardness reduction in the outer 25 μm of enamel specimens tested (McCracken and Haywood, 1995). Knoop hardness values of bovine enamel whitened using a neutral 10% CP product containing carbopol for 21 days were recorded to drop by approximately 77% (Eskelsen *et al.*, 2018). Carbopol is used as a thickening agent and is an acidic polymer that could potentially demineralise the enamel surface, inhibit the formation of hydroxyapatite through its high calcium binding capacity, and ultimately contribute to the reduction in enamel micro-hardness (Basting *et al.*, 2005).

2.3.2 Changes in Enamel Roughness

Constituents released by the breakdown of CP or HP (Figure 1) following a whitening procedure create porosities, grooves, and cracks in enamel, making it rough and more susceptible to extrinsic staining when measured *in vitro* (Shannon *et al.*, 1993; Pinto *et al.*, 2004; Tredwin *et al.*, 2006; Eva *et al.*, 2013). However, replicating this *in vivo* has not provided consistent results. For instance, while post-whitened enamel was up to one-third rougher according to Environmental Scanning Electron Microscope measurements after canine (dog) enamel was treated using 10% and 20% CP, and 25% HP concentrations (El Halim, 2012) a randomised clinical trial in humans revealed no significant difference in enamel roughness following the application of 38% HP and 35% CP (Cadenaro *et al.*, 2008). One potential explanation for this disparity is that in the latter study, (Cadenaro *et al.*, 2008), roughness was measured indirectly using a two stage polyvinyl siloxane impression of the whitened enamel surface, which was then analysed using non-contact profilometry. This could possibly increase the risk of error and affect the accuracy and repeatability of results as a consequence (Jaturunruangsri, 2015).

Enamel roughness is affected by the concentration and pH of the applied whitening gel. A number of studies comparing between whitening products with a variety of pHs used, ranging between 3.2 and 10.8 (Sun *et al.*, 2011), (Eva *et al.*, 2013), (Trentino *et al.*, 2015), (Sa *et al.*, 2012a) revealed an increase in roughness in whitened enamel as whitening agent concentration increases and pH decreases. The thickening agent, carbopol, commonly used in whitening products is acidic and ionic in nature and is derived from carboxylic acid. It has been reported that carbopol in whitening products causes an increase in enamel roughness in comparison to products containing natrosol, a cellulose-based non-ionic polymer, thickening agent (Silva *et al.*, 2018). Others, however, report a greater impact of the application time in comparison to the concentration of the whitening agent applied (Majeed *et al.*, 2008; Grobler *et al.*, 2009). In essence, prescribing non-acidic whitening agents with low concentrations, for a relatively short duration would minimise harmful side effects of whitening agents.

2.3.3 Enamel surface loss

Dental enamel is highly permeable to peroxides (CP and HP), and the penetration depth of whitening agents across enamel, dentine, reaching to the pulp chamber is directly proportional to the duration of application and concentration of the whitening agent (Bharti and Wadhwani, 2013). Many whitening agents have an acidic pH, creating an erosive environment, and contributing to the loss of enamel inorganic matter (Demarco *et al.*, 2011). Friction tests using a tribometer, showed that tooth surface loss in bovine enamel exposed to acidic HP whitening agents (pH 2.7 to 3.9) were 2-3 times the level of loss caused by neutral HP (pH 7.1) (Mundra *et al.*, 2015). According to laser induced fluorescence, the depth of destruction of inorganic matter in enamel following whitening with 30% HP for 60 minutes was up to 1000 μm, and was confined to the external surface of enamel in direct contact with the 30% HP (Jiang *et al.*, 2008). The outermost surface of enamel is the aprismatic enamel, (Públio *et al.*, 2016). This aprismatic structure is highly mineralised and therefore more resistant to demineralisation (Karadas *et al.*, 2014). In *in-vitro* studies, enamel specimens are usually lapped and polished to create a flat surface for experimentation. This removes aprismatic enamel, and exposes the weaker and less mineralised prismatic enamel to testing, therefore, results might be an overestimation of what would happen in real life (Karadas *et al.*, 2014). Incorporating the aprismatic enamel in a laboratory experiment to closely resemble the clinical situation is challenging; it is naturally present in different thicknesses between individuals and within different tooth sites, making it nearly impossible to standardise (Baumann *et al.*, 2015).

Light activated whitening has been shown to be more aggressive than whitening with no light activation (Kugel *et al.*, 2009; Alomari and El Daraa, 2010; Kossatz *et al.*, 2011; Coceska *et al.*, 2015). For instance, diode laser assisted whitening using 30% HP was reported to cause significantly greater enamel damage in the form of mineral loss and loss of interprismatic enamel, when compared to whitening using 40% HP with no light activation source (Coceska *et al.*, 2015).

In an attempt to improve upon whitening agents and minimise surface loss, the addition of casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) as a remineralisation agent proved to stabilise the level of calcium and phosphate in saliva, therefore, enhancing its buffering capacity (Bayrak *et al.*, 2009; George *et al.*, 2015). The significant benefit remineralising agents have, proven by various clinical and laboratory studies, justifies the strong recommendation for their use during or after tooth whitening (Arakawa *et al.*, 2002; Singh *et al.*, 2010; Imamura *et al.*, 2013; Coceska *et al.*, 2015; Low *et al.*, 2015).

2.3.4 Colour change

The effects of dental whitening agents depend upon pH, environmental temperature, added catalysts, and choice of light activation source (Sulieman, 2004). According to a recent systematic review, 10% CP showed similar whitening efficacy with lower risk of tooth sensitivity when compared to whitening using greater concentrations (De Geus *et al.*, 2018). Whitening agents take 5-15 minutes to penetrate enamel, cross the dentine layer, and ultimately reach the pulp (Mccaslin *et al.*, 1999; Haywood and Al Farawati, 2019), and as the rate of colour change is reached, the additional increase in concentration would only cause an increased risk in tooth sensitivity and gingival irritation (Matis, 2003; Haywood and Al Farawati, 2019).

Controversy in relation to the relevance of light activation sources to the whitening process is still evident. According to a review published in 2014, the use of light activation sources have no impact on the whitening efficacy or in accelerating the whitening process (Baroudi and Hassan, 2014). Conversely, results of a randomised clinical trial showed that halogen light significantly improved the level of whitening compared to laser (Polydorou *et al.*, 2013). Whitening was performed using 38% HP for a maximum of four 15 minute intervals until teeth were lightened by six Vita® shade tabs. Dominguez *et al.*, additionally, claims that the light activation source is more relevant to the whitening process than the choice of whitening agent, as whitening using 35% HP activated using light-emitting diode (LED) produced the best whitening result in comparison to laser or halogen (Dominguez *et al.*, 2011).

Colour stability depends greatly on diet and smoking habits as they contribute to the development of extrinsic stains (Karadas, 2015). In a study comparing smokers and non-smokers, results revealed the same level of whiteness a week after tray-based tooth whitening, however, a month later smokers showed darker teeth than non-smokers, indicating the same initial results but different long term whitening stability (de Geus *et al.*, 2015).

In cases of intrinsic staining such as dental fluorosis, enamel microabrasion prior to whitening showed great long term success in three case reports with 11, 20, and 23 year follow ups (Sundfeld *et al.*, 2014). The process of microabrasion eliminated the porous enamel subsurface which entraps stains and causes light scattering; allowing the whitening agent to reach deeper into enamel and produce better whitening results than whitening protocols not including microabrasion (Celik *et al.*, 2013). Bristo *et al.*, proposed abrading enamel surface using a fine diamond bur under water cooling for 5-10 seconds, followed by a 60 second prophylaxis using a 6.6% hydrochloric acid slurry with silicon carbide microparticles. This technique increased the degree of penetration of whitening agents, improving whitening efficacy and long term stability (Briso *et al.*, 2014).

2.3.5 Dental Sensitivity

Whitening induced tooth sensitivity is poorly understood in the literature (Perdigão *et al.*, 2004). Some believe that it is caused by high concentrations of whitening agents, leading to higher levels of by-products released and diffused through dentinal tubules (Eva *et al.*, 2013; West *et al.*, 2013; Chemin *et al.*, 2018). Others attribute sensitivity to the glycerine carrier used in most whitening gels, as its hydrophilic nature causes dehydration of the tooth structure (Leonard Jr *et al.*, 1997; Majeed *et al.*, 2015b).

A relationship between post-whitening tooth sensitivity and the presence of enamel craze lines (i.e. enamel infractions) was studied in a non-randomised controlled clinical trial. The study included 460 teeth (49% of teeth had enamel craze lines) which were subjected to in-office whitening using 15% HP. Results showed 15% of teeth with craze lines and 11% of teeth with no craze lines presented with post whitening tooth sensitivity, indicating a positive but weak correlation between the presence of enamel craze lines and tooth sensitivity (Özcan *et al.*, 2014).

Others report that lower concentrations of whitening agents applied for prolonged periods (10% CP for a total treatment time of 112 hours; 8hrs/day for 14 days) are significantly more harmful to enamel and would consequently cause higher sensitivity levels than high concentrations applied for a short duration (45% CP for a total treatment time of 7 hours; 30 min./day for 14 days) (Majeed *et al.*, 2008). Soares *et al.* reported that whitening using 35% HP for 5 minutes and 17.5% HP for 45 minutes similarly produced significantly less damage to enamel compared to 35% HP applied for 45 minutes, concluding that post whitening sensitivity is dependent upon both whitening duration and concentration (Soares *et al.*, 2014b). Therefore, in an attempt to minimise harmful side effects inflicted by high whitening agent concentrations, researchers have investigated the benefits of separating whitening cycles (de Paula *et al.*, 2015), reducing whitening cycles from 3x15 minute to 2x15 minute cycles (Kose *et al.*, 2016), and incorporating sugar-free gum containing CPP-ACP to reduce post whitening sensitivity.

Attempts to minimise the severity of post whitening sensitivity through the incorporation of desensitising agents have been reported. These attempts, however, did not reduce the risk or severity of tooth sensitivity post whitening (Rezende *et al.*, 2019). According to a randomised triple blind clinical trial, post whitening sensitivity did not significantly differ after whitening using a 10% CP product with 3% potassium nitrate and 0.2% sodium fluoride in comparison to a desensitising free 10% CP whitening product (Maran *et al.*, 2018). In addition, a randomised controlled clinical study revealed no significant difference in sensitivity levels after chewing a pack of 12 sugar free CPP-ACP containing gum for 10 min/hr for a duration of 12 hours upon review 24 hours after whitening using 15% HP (Tang and Millar, 2010). A similar study tested

the effect of chewing 5 pieces of sugar free CPP-ACP containing gum for 10 min/day for one week prior to tooth whitening using 30% HP. Results revealed no reduction in post-whitening sensitivity levels (Henry and Carkin, 2015). A cross-sectional clinical study by Pereira *et al.* tested the buffering capacity caused by gum chewing through stimulating salivary flow using sugar free gum with and without CPP-ACP. Results showed no difference between sugar free gum with and without CPP-ACP (Pereira *et al.*, 2016). The absence of significant results after chewing CPP-ACP containing gum might be attributed to its very low concentration, which was reported to be 0.6% (Henry and Carkin, 2015).

2.4 Cyclical whitening

Understanding the impact of repetitive whitening on enamel is important. As mentioned previously, adverse effects of whitening agents depend on the technique followed, the concentration of whitening agent, and the duration of treatment. Repeated whitening was reported to cause adverse effects, ranging from demineralisation and formation of enamel defects to a more serious side effect such as hyperkeratosis, hyperplasia, and dysplasia (Goldberg *et al.*, 2010). Concerns were expressed in the literature regarding abuse of whitening products, as it has been reported that repeating a whitening cycle using 35% HP one week later, significantly reduces enamel microhardness (Zanet *et al.*, 2011).

In a clinical study, periodontally compromised teeth scheduled for extraction were whitened using 10% CP for 14, 21, and 90 days. Scanning electron microscopic images showed demineralisation of enamel and exposure of prisms after 14 days, with a deeper level of mineral loss leading to exposed prisms, down to enamel rods and frequently to dentine after 90 days (Alqahtani, 2014).

One must, however bear in mind differences between whitening duration and cyclic whitening. Extending the duration of whitening has been predominantly tested in the literature, revealing damaging effects on enamel's chemical composition, physical behaviour, in addition to the overall systemic effects of continuous digestion of small amounts of HP or CP (Goldberg *et al.*, 2010; Alqahtani, 2014; Castro *et al.*, 2015). Cyclic whitening, on the other hand, has been rarely investigated. There are no published studies relating to the monthly repetition of a commonly prescribed tray-based whitening protocol using 10% CP for 2 weeks, for instance, or any other protocol for that matter. The time interval in between whitening cycles might possibly restore the enamel to its baseline values, or similar to reported results of longer whitening durations, long term, repeated whitening of enamel might lead to permanent damage.

2.5 Bovine enamel microstructure

The increasing difficulty in obtaining human enamel (HTA, 2004), led scientists to search for natural and synthetic alternatives, such as bovine enamel, ovine enamel, and hydroxyapatite discs, to be used as specimens in laboratory studies (Rechmann *et al.*, 2017). The reported difficulty was attributed to the fact that most extracted teeth have extensive carious lesions or other defects. Furthermore, the curved nature of human enamel is an additional challenge, as the inability to create large flat surfaces with uniform thicknesses required for some laboratory tests limited its use. The inability to control the source and age of the collected enamel specimen was a reported problem, as it created more confounding factors that have a large impact on the end result of the study (Yassen *et al.*, 2011).

Bovine enamel is the preferred alternative, being easier to obtain, and handle due to its larger size, which provides the added benefit of creating more than one specimen per tooth (Laurance-Young *et al.*, 2011). To date, literature reports diverge when comparing human to bovine enamel; some confirming their histochemical and anatomical similarity (Nakamichi *et al.*, 1983), similar calcium and carbonate levels and fluoride uptake, and similar responses to whitening (Yassen *et al.*, 2011). Others report bovine enamel as the least mineralised specimen when compared to human enamel and pure hydroxyapatite, having a 1.57 Ca/P ratio, while human enamel and hydroxyapatite have 1.61, and 1.67 Ca/P ratio respectively (Rechmann *et al.*, 2017). If this is the case, it is logical to assume that results from a study that uses the less mineralised bovine enamel, after being exposed to an erosive challenge for example, can lead to an overestimation of findings. This was not the case however, in a study where human enamel and bovine enamel behaved in a similar manner with no significant differences in microhardness values, with a recorded knoop hardness of 265 in human enamel and 253 in bovine enamel, after being exposed to orange juice (pH= 3.74) for 10 minutes, 4 times a day for 10 days (Turssi *et al.*, 2010). Furthermore, although similar roughness average (Ra) readings between eroded bovine and eroded human enamel (human 0.23μm, bovine 0.20μm) has been reported, Field *et al.*, stated that profilometric bearing parameters which record surface roughness changes such as the roughness of surface peaks (Rpk), the roughness of surface valleys (Rvk) etc., were significantly different between bovine and human enamel at baseline and post erosion (Field *et al.*, 2013). In fact, surface loss was greater in human enamel than in bovine enamel. This was attributed to the different prismatic/inter-prismatic proportions, revealing greater proportions of inter-prismatic enamel in bovine specimens, which is apparently more resistant to acidic attacks than prismatic enamel (Xiao *et al.*, 2009).

White *et al.*, on the other hand, reported that in an acidic environment with exposure times, between 1 to 60 minutes, bovine enamel surface loss was 30% faster than human enamel,

according to optical profilometry readings (White *et al.*, 2010). Additional studies supporting these results, reported significantly higher calcium ion release in bovine enamel, according to UV spectrophotometery, when compared to human enamel, under altered pH values (Camargo *et al.*, 2006). Additionally, microradiographic readings revealed lower levels of mineral loss in eroded human enamel in comparison to bovine enamel (Meurman and Frank, 1991). The variation in the reported behaviour between human and bovine enamel in the literature is simply due to the fact that enamel microstructure varies according to its location and depth within the tooth from which it was sampled, thus, definitive comparisons are difficult to formulate (Radlanski *et al.*, 2001; Laurance-Young *et al.*, 2011).

Human and bovine enamel are more similar than different according to the current literature, which provides enough justification for substituting human enamel with bovine enamel in laboratory and *in-situ* studies (Yassen *et al.*, 2011; Soares *et al.*, 2016b).

2.5.1 Effect of storage solution

The effects of storage solutions on harvested dental specimens to be used for *in-vitro* studies have been extensively studied, in order to find a storage medium that disinfects and maintains the structural integrity of specimens as when they were initially obtained (Reena *et al.*, 2011). Loss of microstructural integrity due to chemical interactions between the storage solution and human enamel was noted within the literature, revealing a shift in the levels of Ca, Na, P, and K in specimens stored in distilled water with 2% glutaraldehyde, artificial saliva, phosphate buffered saline with 10% formalin, phosphate buffered saline with 0.1% thymol, and saline, for 45 days and for 90 days (Secilmis *et al.*, 2013). The structural similarities between human and bovine enamel helps predict the response of one when the other is tested (Nakamichi *et al.*, 1983). Commonly used storage solutions are presented in Table 1.

Storage Solution	Studies
Artificial saliva	(Worschech *et al.*, 2003; Göhring *et al.*, 2004; Pinto *et al.*, 2004; Earl *et al.*, 2011; Borges *et al.*, 2015; Llena *et al.*, 2017; Tabatabaei *et al.*, 2017)
0.1-0.5% Thymol	(Muraguchi *et al.*, 2007; Lima *et al.*, 2008; Delfino *et al.*, 2009; Xu *et al.*, 2011; Sa *et al.*, 2012a; Moreira *et al.*, 2013; Berger *et al.*, 2014)
0.5%-1% Chloramine-T	(El-Din *et al.*, 2006; Mondelli *et al.*, 2009; Sabatoski *et al.*, 2010; Meireles *et al.*, 2012; Pirolo *et al.*, 2014; Karadas and Hatipoglu, 2015; Lee *et al.*, 2016a)
2-10% Formalin	(Sanae Shinohara *et al.*, 2001; Camargo *et al.*, 2007; Borges *et al.*, 2012b)

Table 1 Shows commonly used storage solutions (type and concentration) as reported published studies.

An ISO report recommends cleaning and pumicing teeth immediately after extraction, then storing samples in distilled water or 0.5% chloramine-T for a minimum of 1 week (Humel *et al.*, 2008). Chloramine-T is a disinfecting N-chlorinated compound, chemically similar to the O-chlorinated sodium hypochlorite, however, differs in its role as a collagen preservative (NCBI, 2017). Another commonly used storage solution, formalin, is used for disinfecting, preserving, and fixing live tissue for microscopic and histological uses, by cross-linking the primary amino group with proteins and DNA (Reena *et al.*, 2011). Storage in 0.1% thymol solution, on the other hand, an antiseptic phenol obtained from thyme oil, was reported to create a flat bovine enamel surface (de Melo Maranhão *et al.*, 2009). This was attributed to its great demineralising effect (extending up to 30µm in depth) due to its acidic nature with a pH value of 4.5 (Moura *et al.*, 2004; Secilmis *et al.*, 2013).

The suitability of storage solutions varies according to the type of study conducted. For instance, in hardness studies ionised water and 0.1% thymol did not alter hardness values of bovine enamel in comparison to baseline values (Aydın *et al.*, 2015). Physiological saline, on the other hand, caused significant reduction in enamel hardness values, making it unfavourable in these types of studies (de Melo Maranhão *et al.*, 2009). Results, however, might vary, as storage in 10% formalin was reported to be the most effective storage medium when measuring bond strength in one study (Lee *et al.*, 2007), while significantly reducing bond strength according to another study (Humel *et al.*, 2008). This being the case, these contradictory results must be interpreted with caution, by assessing concentrations of storage solutions and storage durations.

It is vital to appreciate the impact of storage duration, as one study reported the maximum allowed storage time of bovine enamel in 0.2% thymol, 10% formalin, and 0.2% sodium azide, without affecting enamel structural and mechanical behaviour, to be up to 6 months (Santana *et al.*, 2008). Another study, evaluated the impact of 8 storage solutions on human enamel at different time intervals for up to one year, using 1% chloramine-T, 10% formalin, 10% buffered formalin, 0.02% thymol, 0.12% chlorhexidine, 3% sodium hypochlorite, artificial saliva, and saline, all stored at 4°C (Kaul *et al.*, 2014). Results were compared against frozen teeth stored at -20°C, revealing significant reductions in mineral content and fluorescence response in all 8 groups, according to laser fluorescence readings, with maximum reduction noted during the first 30 days of storage. The frozen group, exhibited no significant change in fluorescence response, proving the efficiency of freezing in storing teeth without chemically altering their composition.

To create a storage solution structurally close to natural saliva, formulations of artificial saliva (AS) were proposed (Göhring *et al.*, 2004; Earl *et al.*, 2011). Storing enamel in AS following

exposure to an acid challenge was reported to significantly increase mineral gain in eroded enamel, according to readings obtained using contact microradiographs (Kielbassa *et al.*, 2001). Studies also show that AS, as a storage medium has no significant effect on enamel colour and hardness values, in fact, enamel samples whitened and stored in AS exhibited no significant changes in hardness compared to BL values and the degree of colour change was similar to that recorded in samples stored in natural saliva (Zeczkowski *et al.*, 2015).

According to Gal *et al,* manufacturers mainly focus on the mineral composition of artificial saliva, neglecting the importance of simulating salivary viscosity (Gal *et al.,* 2001). The level of viscosity is determined by levels of glycoproteins present, which control the diffusion rates and reaction rates, thus, making it essential in recreating natural saliva. This possibly explains the large precipitation of minerals reported in one study when specimens were stored in artificial saliva for 90 days (de Melo Maranhão *et al.*, 2009). Although mineral percentages might resemble those found in natural saliva, the absence of a mineral diffusion mechanism and exchange of solutes governed by saliva viscosity, might have led to the accumulation of minerals. Therefore, evidence shows that storage in human saliva leads to higher levels of remineralisation in comparison to artificial saliva, possibly leading to an overestimation of the effects whitening agents have on enamel (Attin *et al.*, 2009).

To date, controversy regarding the best storage solution still exists. The uncertainty of the impact these solutions have on dental tissues and contradictory results reported, make decisions more challenging. Using solutions with long track records in *in-vitro* studies might be the safest option, at least until an ideal storage medium is discovered.

2.6 Enamel remineralising agents

2.6.1 Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP)

In an attempt to accelerate the mineral uptake and remineralisation process of enamel, reduce dentine hypersensitivity, and even counteract the harmful effects of xerostomia, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) (Al Batayneh, 2009) has been added to a number of different dental products, such as toothpastes (Gjorgievska and Nicholson, 2009), mouth rinses (Reynolds *et al.*, 2003) restorative materials (Mazzaoui *et al.*, 2003; Zalizniak *et al.*, 2013) chewing gums (Iijima *et al.*, 2004; Morgan *et al.*, 2008), and more recently in tooth whitening products (Neuhaus and Lussi, 2009; Farooq *et al.*, 2013; Somasundaram *et al.*, 2013).

CPP-ACP was introduced in 2002 as a 10% CPP-ACP paste (GC Tooth Mousse™, GC Corporation, Melbourne, Australia), which is derived from milk protein 'casein', and due to its high affinity it binds and aggregates with calcium and phosphate ions in an amorphous state and chemically bonds to hydroxyapatite present in hard tissue, maintaining the saturation of calcium and phosphate ions, thus, hindering the demineralisation process caused by bacterial or erosive attacks (Somasundaram *et al.*, 2013).

GC Tooth Mousse™ proved to successfully accelerate the remineralisation process of enamel and reduce the likelihood of mineral loss and white spot lesion formation (Rao and Malhotra, 2011, Memarpour et al., 2015, Jefferies, 2014, Somani et al., 2014). Twice daily use of CPP-ACP significantly affected the remineralisation process of post orthodontic white spot lesions when combined with a 1000 ppm fluoride toothpaste (Singh et al., 2016). The significant reparative capacity of CPP-ACP in regards to remineralisation and structurally strengthening dental hard tissue is supported by the literature (Jayarajan et al., 2011, Savas et al., 2016, Reynolds, 2008, Reynolds et al., 2008, Reynolds, 1997, Reynolds, 1998, Ceci et al., 2015). There is a strong correlation between the application of (CPP-ACP) and the resultant recovery in enamel micro-hardness 10 days and 15 days following its application according to measurements obtained by Vickers micro-hardness tests (Palaniswamy et al., 2016, Yengopal and Mickenautsch, 2009, Grobler et al., 2011, Peric et al., 2014, Bayrak et al., 2009b, Ahmadi Zenouz et al., 2015).

In an attempt to maximise whitening efficiency while minimising sensitivity, the combined effect of using CPP-ACP with tooth whitening agents was tested by either applying 10% GC Tooth Mousse™ on enamel before or after whitening. This resulted in a significant reduction in post-whitening sensitivity with no impact on whitening efficacy (Borges *et al.*, 2011b; de Vasconcelos *et al.*, 2012; Alkhtib *et al.*, 2013; Po and Wilson, 2014). The application of CPP-

ACP paste before, after, or before and after whitening with 35% HP has additionally prevented significant hardness and roughness changes in bovine enamel following a 14 day whitening cycle (Gama Cunha *et al.*, 2012). Results were confirmed by another whitening study using human enamel samples, revealing a significant improvement in enamel remineralisation and recovery of microhardness values upon the application of CPP-ACP paste (GC Tooth Mousse™) after whitening (Coceska *et al.*, 2015; Penumatsa *et al.*, 2015).

According to a clinical study, roughness values of whitened enamel decreased by 50% after being remineralised using CPP-ACP, nano-hydroxyapatite, or NovaMin® for 5 minutes after each whitening cycle (da Rosa *et al.*, 2015). According to SEM images, a uniform layer of these bioactive pastes enhanced the remineralisation process and reparative capacity following the structural damage caused by whitening, presenting as depressions and prismatic exposures, therefore creating a smoother and more uniform surface.

The long track record of CPP-ACP used before and after whitening treatments demonstrate its effectiveness in reducing negative side effects caused by peroxides, however, differences in its remineralising effect in enamel whitened using different pH values of HP has not been reported in the literature.

2.6.2 Nano-hydroxyapatite

Hydroxyapatite is a calcium phosphate compound that can be either synthesized or extracted naturally from bovine teeth or bones for example (Barakat *et al.*, 2009). In an attempt to maximise the benefits of dental products such as toothpastes, scientists incorporated nano-hydroxyapatite (nHA) particles for remineralisation, ability to reduce *Streptococcus mutans* virulence adsorption, and to remove extrinsic stains by virtue of its abrasive nature (Kani *et al.*, 1989; Huang *et al.*, 2009; Tschoppe *et al.*, 2011).

Enamel building blocks are 97% HA crystals which are 20-40nm in size. If an attempt to repair enamel was undertaken after an erosive or abrasive attack, it would be logical to do so using nano-sized HA (nHA) particles as they have proven to self-assemble, creating enamel like structures in aqueous solutions, which is a unique nanoscale advantage (Figure 2) (Robinson *et al.*, 2004; Tao *et al.*, 2007). Li *et al.*, reported the strong affinity, biocompatibility, and reparative capacity of 20 nm sized artificial nHA applied to eroded human enamel, which was not evident following the application of ACP or larger sizes of HA particles (Li *et al.*, 2008). This could be explained by the high surface to core atoms present in nano-particles, meaning that in nano-sized particles there are more surface atoms with fewer bonds than atoms located deep into the core. This creates a more reactive particle with a higher potential to create new and strong bonds (Binns, 2010). For this reason, the larger number of unbound surface atoms in a nano-particle, in comparison to micro- or macro- scales, allow the creation of more bonds with surrounding structures, therefore, forming a more strongly adhered compound (Tao *et al.*, 2007; Binns, 2010).

Multiple clinical studies have been conducted using nHA toothpastes since the 1980s. A three-year clinical study done in 1983 revealed a reduction in caries incidence by up to 56% in 181 school children. The programme was conducted in three primary schools, and all three schools were provided with toothbrushes. Toothpastes with nHA were given to one school, while the other two schools were given a control toothpaste with no nHA additive. Upon comparison of DMFT scores at baseline and at three years, the use of 5% nHA toothpaste once a day significantly reduces the incidence of caries (Kani *et al.*, 1989). More studies supporting the use of nHA in dental products were published afterwards. For instance, in comparison to fluoride, nHA containing pastes proved to be more effective in occluding dentinal tubules, thus, reducing dentine sensitivity, and in restoring roughness to pre-whitened conditions according to images obtained by scanning electron microscopy (SEM) and scanning probe microscopy (SPM) (Kawamata *et al.*, 2004; Ohta *et al.*, 2007; Amaechi *et al.*, 2015; Low *et al.*, 2015).

An additional reported advantage of nHA is its ability to shift the oral flora to a more favourable condition. Saliva sampling collected to determine bacterial ratios before and after a 5 min.

application of nHA paste on 19 subjects revealed that it adheres to tooth and plaque surfaces and selectively adsorbs harmful bacteria such as *Streptococcus mutans* and *Porphyromonas gingivalis*, which in addition to reducing the incidence of caries, may potentially help prevent the occurrence of plaque related periodontal disease by smoothing plaque retentive rough enamel surfaces (Arakawa *et al.*, 2002; Arakawa *et al.*, 2010; Fujimaru *et al.*, 2015). In contrast, nHA was unable to restore roughness values in 15 enamel specimens following orthodontic debonding to pre-treatment levels. The serum with an undisclosed concentration of nHA (n-HAP Repairing Serum; PrevDent International BV, Netherlands) was applied for 2-3 minutes then rinsed off after 20 minutes. The procedure was repeated for 10 days, and revealed no significant improvement in enamel roughness according to AFM measurements (Ajami *et al.*, 2016).

Significant improvements following the application of nHA required 90 minutes (Arakawa *et al.*, 2002), 2 weeks (Low *et al.*, 2015), 2 to 5 weeks (Tschoppe *et al.*, 2011), and 2 months (Fujimaru *et al.*, 2015), which supports the argument that the application duration of nHA is an important factor to consider.

Regardless of the early development of nHA containing products, and the applications, benefits, and limitations reported in the literature to date, the amount of research on nHA is underwhelming, and overshadowed by other materials that have similar mechanisms of action. This leaves room for research to potentially improve currently used products with nHA additives.

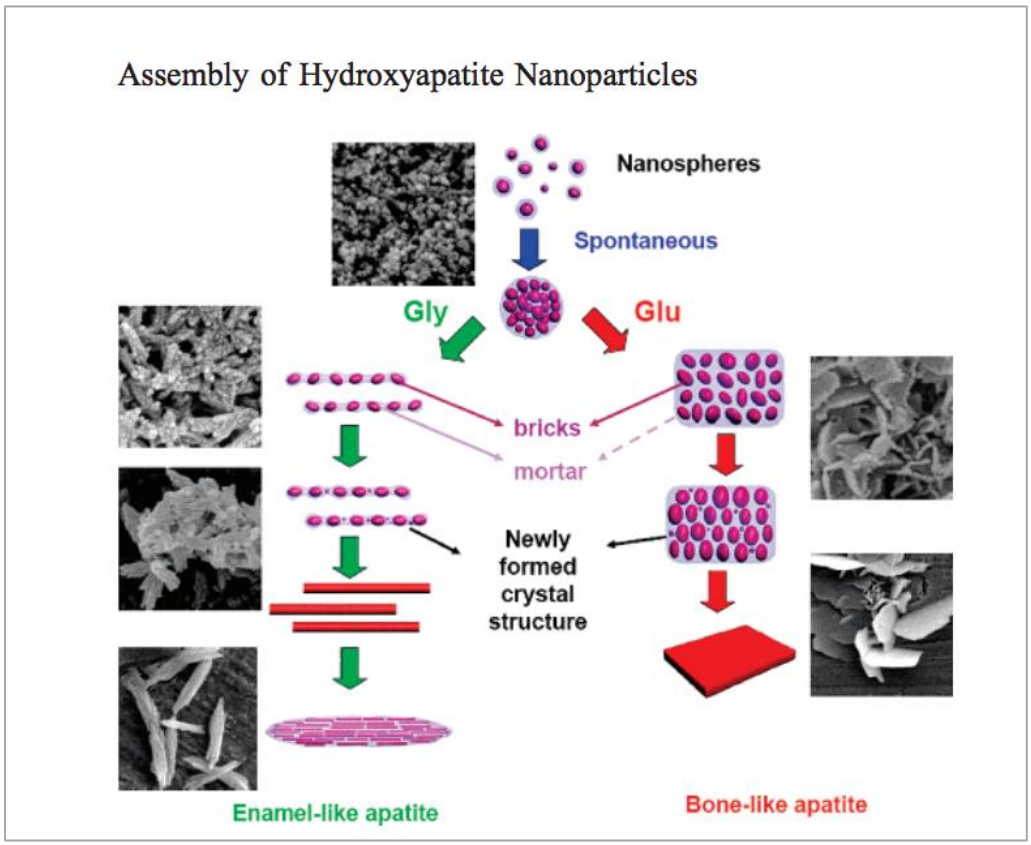


Figure 2 Shows a schematic model and SEM images demonstrating the interaction and aggregation of hydroxyapatite nano-spheres (purple) with ACP (blue) under the control of biological elements such as glycine (Gly) and glutamate (Glu). The newly formed crystallised(nHA) bricks are then organized and arranged into a linear or plate assembly and fuse to form a single HAP crystal to be used as a building block. Source: Tao et al. 2007.

2.7 Impact of dietary acids on whitened enamel

The effects of dietary acids on whitened enamel are reported to include significant reduction in hardness, enamel mineral loss, and formation of surface porosities (Yeh *et al.*, 2005; Zanet *et al.*, 2011). Whitened bovine enamel exposed to beverages containing dietary acids with pH values ranging between 2.75 and 3.29, for 7, 14, and 21 days were structurally more vulnerable to erosive attacks when compared with un-whitened enamel (Zanet *et al.*, 2011; de Araujo *et al.*, 2013). Repeated exposures to HP and dietary acids negatively affected enamel hydroxyapatite crystals, through dissolving the calcium ions and mineral crystals. This was confirmed by de Araujo *et al.*, who reported higher levels of mineral loss in enamel whitened for 6 hours/daily for a total of 3 weeks, using 10% CP, followed by immersion in a cola soft drink for 1 hour after each whitening cycle in comparison to a control group stored in artificial saliva for the duration of the experiment (de Araujo *et al.*, 2013).

In an effort to compare the impact of dietary acids and whitening agents on enamel, enamel exposed to either 38% HP or orange juice (Fawad, 2015) exhibited a statistically significant difference in hardness (from 161 at BL to 156.8 after exposure) in the group exposed to orange juice, while whitened enamel exhibited no difference in hardness. A similar study comparing hardness values and surface topography of enamel specimens exposed to either 6% HP (pH of 5.5) or orange juice (pH of 3.8) revealed a 84% hardness reduction and significant topographical changes in enamel exposed to orange juice, with no significant difference observed in the whitened group (Ren *et al.*, 2009). This being the case, and since the cumulative effects of whitening and dietary acids are rarely studied, it is likely that the combined effect of HP and dietary acids are more harmful to enamel than their separate effects.

2.8 Whitening stability and dietary stain absorption

Patients are advised to minimise or eliminate dietary components that may cause enamel staining before, during, and after any whitening treatment, and for that reason, investigations on the susceptibility of whitened enamel to staining have been abundantly reported. Whitened enamel has a higher tendency for stain absorption, when compared to un-whitened enamel (Ghavamnasiri *et al.*, 2006; Karadas *et al.*, 2014). The effect of whitening on enamel roughness was mostly reported to lead to the enamel surfaces becoming rougher after whitening, with a higher susceptibility to stain absorption and retention compared to control groups (Azrak *et al.*, 2010; Dent *et al.*, 2010; El Halim, 2012).

Immersion of bovine enamel samples in red wine for 48 hours, either immediately, 24 hours, or one week after whitening with 35% HP exhibited similar levels of stain uptake regardless of the time frame separating whitening and staining treatments (Berger *et al.*, 2008). Additionally, an *in-situ* study where small enamel slabs were mounted in intra oral devices and whitened using 35% HP and then stained either immediately or 7 days after whitening, showed no significant differences in enamel whiteness indices after being exposed to coffee (Mori *et al.*, 2015).

The immersion of human and bovine enamel in coffee between whitening cycles using 16% CP had no significant impact on the shade of whitened enamel (Attia *et al.*, 2009). Furthermore, spectrophotometric evaluation of human enamel whitened using 10%, 15%, or 20% CP for 6 hours per day, for 3 days, followed by immersion in coffee or red wine for 15 minutes after each whitening cycle, revealed that during whitening no significant pigment uptake was noted (Côrtes et al., 2013). It has been theorised that storage in artificial saliva and subsequent whitening treatments might reduce or eliminate the effects of coffee and red wine. Upon conclusion of the whitening treatment, enamel was immersed in coffee or red wine, and the colour uptake was measured 7, 15, and 30 days, post immersion. Readings revealed a significant stain uptake in both groups, more so in the red wine group. This was explained by the acidic nature of red wine, causing an increase in enamel roughness and susceptibility to stain uptake.

Colour stability of whitened enamel in a 12-month *in-vitro* colorimetric evaluation revealed a significant increase in L* (lightness values), and decrease in b* (yellowness values) immediately after whitening. There were no observed changes in the a* value, indicating no significant colour alterations along the red-green axis. (Wiegand *et al.*, 2008). Enamel was whitened using 6% HP (Whitestrips™, Procter & Gamble, Egham, UK), 15% CP (llumíné, Dentsply, DeTrey, Konstanz, Germany), or 38% HP (Opalescence Xtra Boost, Ultradent Products, South Jordan, Utah, USA) for 21 days. Colour measurements were obtained immediately after whitening and 3, 6, and 12 months post-whitening. During the observation

period, *L** levels decreased gradually, however, they were still greater at 12 months post whitening than they were at baseline. The reduction in lightness was attributed to the organic constituents in artificial saliva, which were suggested to cause lightness reduction and colour regression. The *b** values, however, were constant throughout the 12-month follow-up period. This was explained by the possible irreversible degradation of organic matter in enamel, leading to a constant reduction in yellowness. In contrast, other studies revealed that overall enamel colour change (ΔE) significantly decreased when comparing readings obtained immediately after whitening to readings obtained 6 months later. At 6 months post whitening a 45% reduction in ΔE was recorded in one study (Matis *et al.*, 1998). According to another clinical study, colorimetric readings of ΔE regression was up to 51% and 65% after one week and 6 weeks post whitening respectively (Matis *et al.*, 2007). The variation in colour relapse observed in clinical studies, was attributed to the frequency of consuming dietary pigments, smoking habits, and the level of oral hygiene (Wiegand *et al.*, 2008).

Differences in findings between clinical and laboratory studies might be caused by the choice of storage solution, the elimination of aprismatic enamel by the lapping and polishing processes, or due to the possible variation in enamel sample microstructure according to its location and depth within the tooth from which it was harvested (Laurance-Young *et al.*, 2011).

2.9 Quantitative and Qualitative assessment of Whitened Enamel

2.9.1 Quantitative assessment

Measuring enamel colour

A thorough colour evaluation includes three key aspects: the colour parameter, whiteness index, and colour difference. This enables the examiner to quantitatively measure the colour under investigation, understand its whiteness level, and how far it is from standard (Luo *et al.*, 2007). These methods have been employed in many studies in the field of dental sciences (Garcia *et al.*, 1993; Luo *et al.*, 2005; Luo *et al.*, 2007), and proved to be effective in measuring colour changes following the application of tooth whitening agents.

The Commission Internationale De L'Eclairage (CIE) parameter variables are L*, a*, and b* which were introduced in 1976 as a three-dimensional representation of colour. L* represents the lightness and darkness expressed by the evaluated colour where (L*=0) is dark and (L*=100) is light. The variable a* evaluates the colour along the red-green axis, and b* evaluates the colour along the blue-yellow axis (Figure 3)(Luo *et al.*, 2007). The delta E value (ΔE) measures the total colour difference in the L*, a*, and b* parameters (Mokrzycki and Tatol, 2011). The CIELAB is the most widely used system in most countries and is a recommended system for colour measurement in dentistry, due to its high precision and accuracy (Seghi *et al.*, 1989; Jha, 2010). Following whitening of enamel, the L* value will shift towards the positive direction, expressing lightness, and the b* and a* towards a more negative value, becoming less yellow and red and more blue and green respectively (Singh *et al.*, 2010).

Other colour measuring parameters used in some industries are the Munsell system and the Swedish Natural Colour system (Judd and Nickerson, 1975). The Munsell system is presented as a circle of hues (colours) divided into 100 equal divisions, chroma (saturation) starting at 0 from the centre of the circle and increasing in value as it moves outward, and finally along an axis perpendicular to the hue and chroma is the value (lightness and darkness) where lightness levels increase moving up and decrease moving down (Figure 4). The Munsell system is widely used in food industry literature and has been recommended by the US Department of Agriculture as a colour measuring system for food quality inspections (Jha, 2010).

The Swedish Natural Colour system was initially proposed by the German physiologist Hering in 1964 (Hering, 1964). Developers claim that through human perception it is possible to accurately identify the magnitude of hues, whiteness, and blackness (Kuehni, 2012). According to the literature, the Natural Colour system proved to be less reliable as standardisation is difficult to apply on a system built on human perception (Ronchi, 2015).

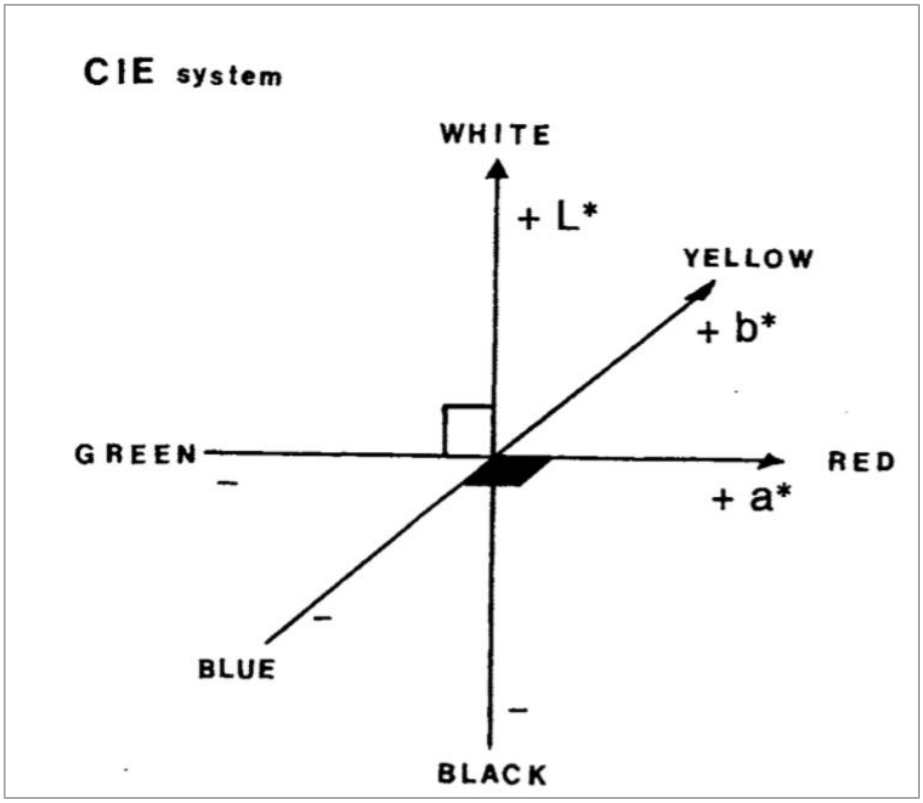


Figure 3 Shows the three dimensional CIE colour system with L, a*, and b* variables. source: Fullerton et al. (1996).*

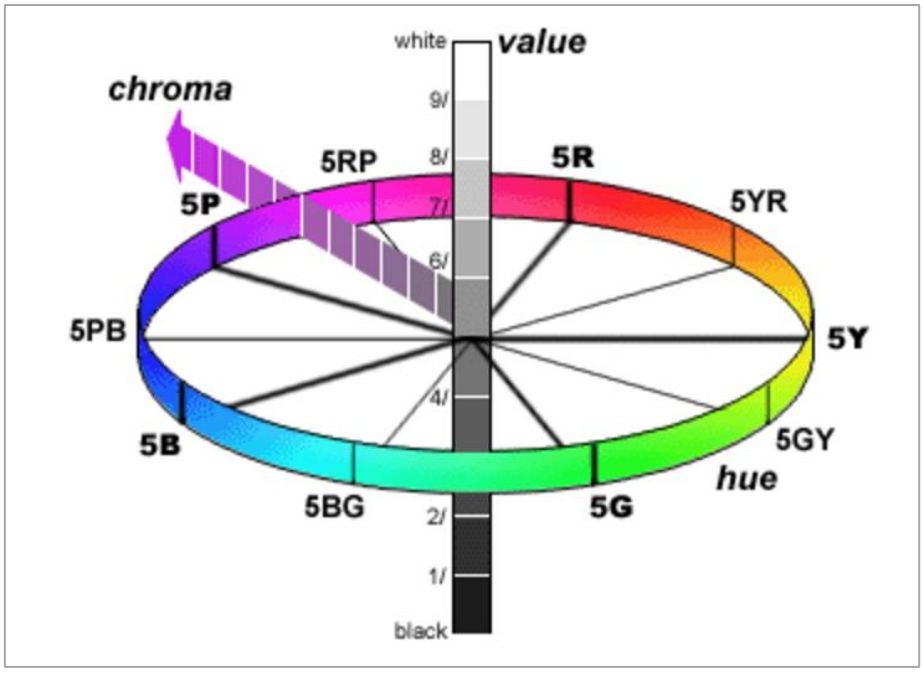


Figure 4 Shows the Munsell colour system, calibrated according to hue, chroma, and value. source: MacEvoy (2005).

A whiteness index measures the level of whiteness of a surface that is white or near white (Joiner *et al.*, 2008). The CIE whiteness index (WIC) is the most commonly used whiteness index and was originally developed to measure the whiteness index in textiles and paint (Luo *et al.*, 2007). The optimized whiteness index (WIO) is a modified version of WIC which is more accurate and reliable as a dental whiteness index (Luo *et al.*, 2005). Finally the third whiteness index W* represent the distance between the colour value from a white point (L*=100, a*=0, b*=0) (Luo *et al.*, 2007; Gulrajani, 2010).

Colour measurements in dentistry are commonly performed using spectrophotometers or colorimeters, as they were reported to provide reproducible and objective readings (Kielbassa *et al.*, 2009; Zarei *et al.*, 2017). Colorimeters are used to measure the amount of absorbance of a specific colour and work only in the range of visible light in the electromagnetic spectrum. Spectrophotometers, on the other hand, measure the amount of transmittance and reflectance of all colours and include measurements of infrared and ultraviolet light (Gulrajani, 2010). Spectrophotometers are among the most accurate colour measuring instruments (Chu *et al.*, 2010). Spectrophotometers contain an optical radiation source, a light dispersion apparatus, a detector, an optical measuring system, and a means converting the detected light into a signal that can be recorded and analysed (Figure 5) (Chu *et al.*, 2010). In comparison to subjective visual shade matching, spectrophotometer readings were reported to be 33% more accurate and provided a more objective match in 93.3% of cases (Paul *et al.*, 2002). Colorimeter readings on the other hand, were reported to show great variability in accuracy among devices, ranging from 67% to 93% (Kim-Pusateri *et al.*, 2009).

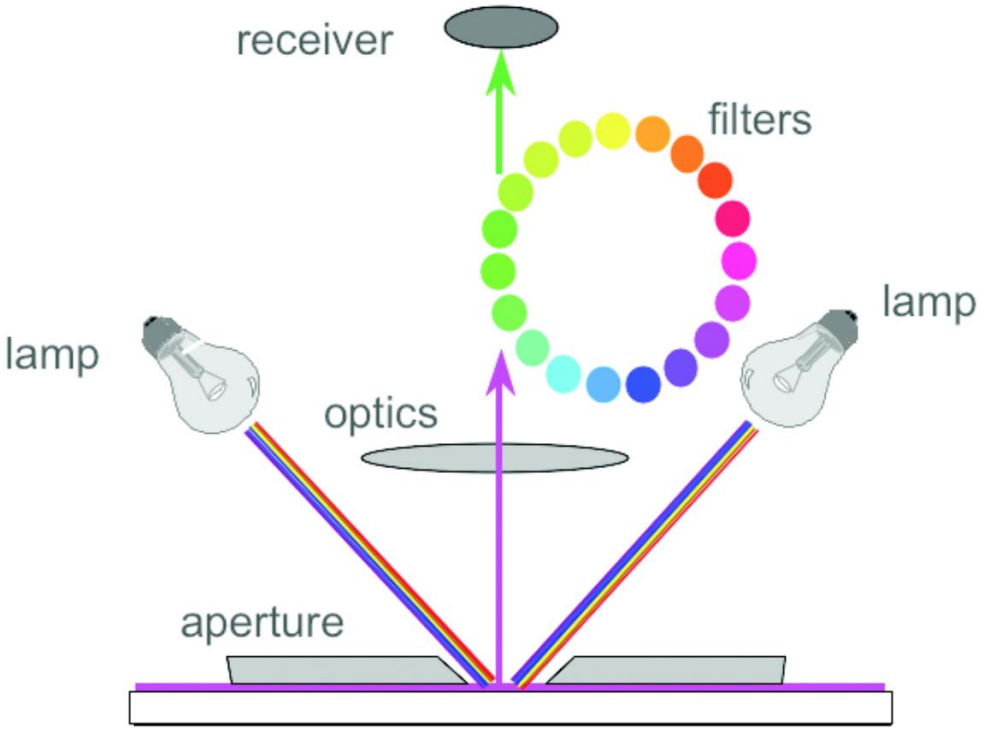


Figure 5 Shows a schematic illustration of colour measurement using Spectrophotometry. Beginning with light emission, which then gets dispersed; passing through an aperture towards the sample. Light reflected from the sample's surface passes through a ring of filters, each of which is sensitive to a different portion of the visible light spectrum. Data received are finally displayed as readings on a digital screen. Source: (X-Rite, 2015).

Digital imaging has been widely used as a means for colour measurement. Capturing an image using a high precision camera followed by processing the captured image using an image processing software has proven to be highly accurate in matching the captured image against a wide range of shade values, and was more reliable when compared to observer's shade matching (Jarad *et al.*, 2005; Oh *et al.*, 2010). This was confirmed by a study conducted in 1999, as digitized images provided accurate and reproducible results upon monitoring colour changes after whitening treatments (Bentley *et al.*, 1999). This was confirmed by Peskersoy *et al*. in 2014, stating that digital imaging and software analysis is an effective objective method in numerically evaluating colour (Peskersoy *et al.*, 2014). However, standardisation of light conditions is essential to accurately compare the before and after images. This can be achieved by using a black box and light ring with standard light intensity, by standardising the height of the tripod used, to ensure images are taken along the same angle, and by ensuring that all background lighting in the room is consistent (Jarad *et al.*, 2005; Polydorou *et al.*, 2008).

The Value oriented-Vita® shade guide is the most frequently used method to visually assess whiteness differences as it is quick, cost effective, and an easy method to convey the selected shade with the laboratory and with the patient (Gulrajani, 2010). The Vita Classical shade guide was first introduced in 1956 followed by the 3D master guide which was introduced in 1998 (Bhat *et al.*, 2011). It is, however, a subjective method and errors in measurement are very likely to occur among assessors, due to differences in light conditions, eye fatigue, experience, and age (Berns and Reiman, 2002; Paul *et al.*, 2002). This led to the introduction of digital versions e.g. VITA Easyshade® (VITA Zahnfabrik, Bad Säckingen, Germany), which according to the literature is an intra-oral spectrophotometer that takes into account reflection, scattering, translucency, and material thickness with a 96.9% reliability (Paravina *et al.*, 2007; Kim-Pusateri *et al.*, 2009).

A number of colour measuring techniques have been proposed to minimise the occurrence of errors during shade matching. Based on the aforementioned, spectrophotometers prove to be the most accurate colour measuring technique, followed by colorimeters, digital imaging, and finally subjective visual shade matching.

Atomic Force Microscopy

The atomic force microscope (AFM) measures surface structure through a quantitative high-resolution imaging process with great accuracy and precision down to the nano-scale (Eaton and West, 2010). It is a commonly used measuring instrument due to its ability to image a wide range of materials from hard ceramics and metals to biological soft samples and DNA. Its imaging process differs from other microscopes as it physically traces the sample surface with a sharp probe (radius<20nm), to build a three-dimensional surface topography.

The main components of an AFM are the control electronics, a computer, and a microscope stage supported on a vibration isolation platform to maximise resolution and minimise noise (Eaton and West, 2010). The stage contains the scanner, a sample holder, and a force sensor. The force sensor can measure very low forces using an optical lever holding the probe on one side and a reflective surface on the other side (Butt *et al.*, 2005). When the probe interacts with an uneven sample surface, the path of a fixed laser beam reflected by the cantilever will change. Changes in the path of the reflected laser onto the four-segment photodetector will determine the surface topography through voltage conversion.

The control electronics are responsible for digitising images taken from the microscopic stage and transferring results to the computer for interpretation (Eaton and West, 2010). The operation concept is based on the ability of the piezoelectric transducer to move the tip towards the sample surface (Butt *et al.*, 2005; Eaton and West, 2010). The force sensor would then detect the force between the tip and the sample, which will be fed back into the feedback control, and a signal will then be generated as a result, and sent back to the piezoelectric transducer to maintain a fixed force between the tip and the sample measured to prevent damage to both. It has been estimated that the few angstroms between the sample and probe tip is being maintained by a strong repulsive force due to an overlap in atomic orbitals. The upward and downward movement of the tip as it traces the sample surface (z) is plotted along with the (x-y) position of the tip across the surface to create a three-dimensional image. The vertical force applied by the tip onto the sample is measured by a force distance curve, which plots the cantilever deflection versus the extension of the piezoelectric scanner (Figure 6).

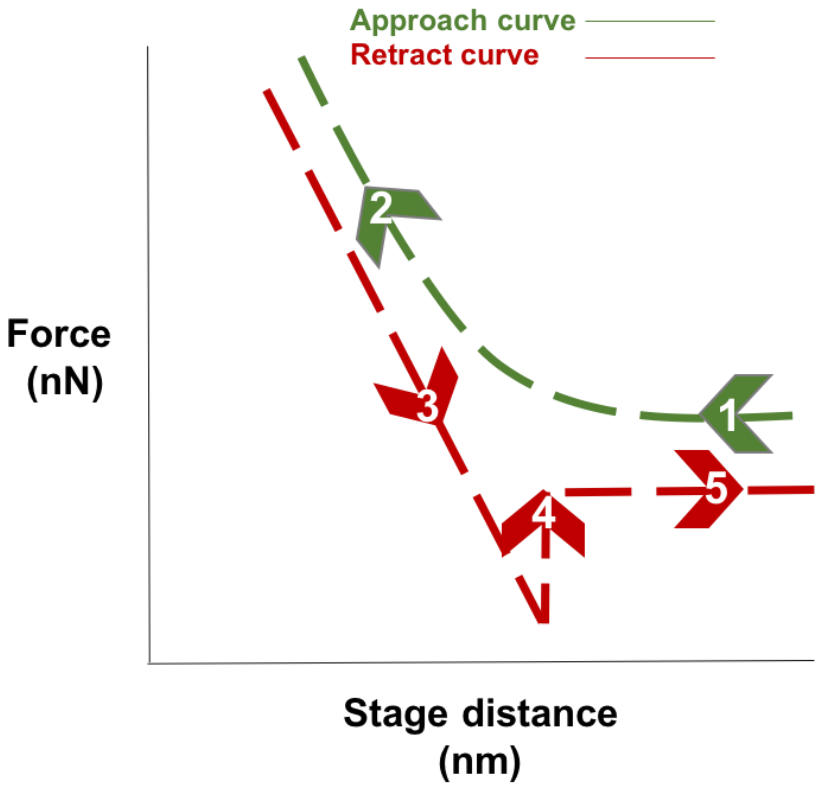


Figure 6 Shows the Force-Distance curve. The probe approaches the sample surface with no resisting force, thus a flat curve is expressed (1), followed by a force exhibited by the probe pushing against the surface (2), then the probe is retracted and pulled off the measured surface (3), a snap off section follows which shows the level of probe sample adhesion (4), and finally the retraction is completed with the probe being fully disengaged shown by the flat curve (5).

The contact mode AFM was invented in 1985 by Binning *et al.*, providing efficient readings on the nano/micro scale by "dragging" the probe across the surface using a cantilever with a probe/cantilever spring constant lower than the spring constant of the force holding sample atoms together (Binnig *et al.*, 1986). As the probe tip traces the sample, measurements are obtained through a probe/sample force constant ranging from 0.01 to 1.0 N/m, which is maintained through a feedback loop. The use of contact mode AFM however, was limited due to difficulty in maintaining atomic imaging stability in addition to the possible damage an applied high lateral force could have on measured surfaces. As a result, oscillation of the cantilever during imaging was proposed and explored, leading to the introduction of tapping mode AFM.

The tapping mode allows the probe to get close enough to the sample to apply detectible short-range forces at or near its resonance frequency and amplitude, ranging from 20nm to 100nm (Zhong *et al.*, 1993). The light tapping of the tip prevents it from sticking onto the measured surface, which was a major problem in the contact mode especially in ambient conditions. The feedback loop in tapping mode, maintains a fixed and constant resonance amplitude, and surface changes are detected by changes to the vibration frequency (Binnig *et al.*, 1986).

The non-contact AFM was introduced in 1994, which succeeded in obtaining atomically resolved stable imaging (Morita *et al.*, 2015). The probe tip does not come in contact with the sample surface, it rather oscillates at or near its resonance frequency, typically 100-400 kHz. The van der Waals forces which are strongest when sample/tip distance is between 1-10 nm, act to decrease the resonance frequency of the cantilever, by this maintaining a constant oscillation amplitude (Gross *et al.*, 2009). The non-contact mode is best used to image hydrophobic samples as any fluids present could encapsulate the probe tip when it comes into contact with the measured surface, resulting in a distorted image (Giessibl, 2003).

The quantitative imaging mode (QI™ mode) is an advanced multiparametric force spectroscopy mode, that records a force distance curve in every imaged pixel, eliminating the need for set-point or gain adjustment while imaging (Casdorff *et al.*, 2017). It takes up to 1µm/s to measure one force distance curve with an image resolution reaching up to $512px^2$ (Chopinet *et al.*, 2013). This imaging mode prevents lateral forces and controls vertical forces applied by the probe, ensuring a non-destructive imaging process for the probe and the measured surface. The QI™ mode is suitable for soft, loosely attached, and sticky samples in addition to samples with sharp edges. From a single image, information on surface topography in addition to mechanical properties of the tested sample can be obtained (Chopinet *et al.*, 2013; Smolyakov *et al.*, 2016). Atomic force microscopy measures roughness of materials with very high resolution, down to the nanoscale, in comparison to stylus and optical profilometers (Bhushan, 2000). Roughness

is measured by calculating the magnitude of all positive and negative deviations presented as peaks and valleys respectively, in relation to a straight baseline (Field *et al.*, 2010). Atomic force microscopes are preferred over profilometers, as detection of surface irregularities is limited by the radius of the stylus/ probe tip (Heurich *et al.*, 2010). Therefore, surface depressions that have smaller dimensions than the profilometer stylus tip, for example, will not be identified; contributing to an underestimation of roughness changes. Furthermore, the use of stylus profilometers is contraindicated in erosion studies, as dragging a stylus tip across an eroded soft surface could lead to the development of surface scratches, resulting in greater or inaccurate roughness readings (Hjortsjö *et al.*, 2010).

Atomic force microscopes have the ability to measure Young's modulus, which indicate material stiffness down to 1nm, by continuously measuring the load and displacement within the tested material as the indenter pushes into the sample (Bhushan and Koinkar, 1994; Hoffman, 2010). AFM is commonly used to measure whitened dental surfaces (Mahringer *et al.*, 2009; de Freitas *et al.*, 2010; Sa *et al.*, 2012b; Soares *et al.*, 2013; Varanda *et al.*, 2013; Khoroushi *et al.*, 2015; Karakaya and Cengiz-Yanardag, 2019), proving to be an efficient and reliable measuring technique.

Energy Dispersive X-ray Spectroscopy

This elemental detection device performs a surface analytical technique where an electron beam in the range of 10-20 keV hit the sample (Ganesh Kumar *et al.*, 2016; Polini and Yang, 2017). The beam will excite an electron in the inner shell, resulting in its ejection from the electronic structure of the element, resulting in an electronic hole. As the electron beam travels through and ionises the sample, two physical events occur as a result: elastic scattering and inelastic scattering (Scimeca *et al.*, 2018). Elastic scattering is caused by a change in the direction of the electron with no associated loss in energy, and it is the main determinant of the shape of the interaction volume. The inelastic scattering, on the other hand, involves the loss of energy with no change in electron direction, and is a major determinant of the size of the interaction volume (Morgan, 1985; Scimeca *et al.*, 2018). As the atoms transition from their ionised state to their ground state, they emit characteristic X-rays from each element within the tested sample surface (Ganesh Kumar *et al.*, 2016). Using a semiconductor detector of a high purity silicon, all X-ray energy emitted from the sample get converted into a pulse voltage, and pulse numbers are counted by a multi-channel pulse height analyser (Harada and Ikuhara, 2013). This process leads to the creation of an energy spectrum (Figure 7) and the position of the peak and its energy determines the element detected, and the area under the peak reflects the number of element atoms present in the area tested (Scimeca *et al.*, 2018).

There are two types of Energy Dispersive X-ray Spectroscopy (EDX) detectors; a normal detector (Be-window type) known to be highly efficient, by detecting X-ray beams through a Be-window which is around 7-mm thick, and an ultra-thin window detector which detects X-rays through a window with a thin plastic film coated with 0.1 mm thick aluminium making it very effective in detecting light elements such as C, N, and O (Harada and Ikuhara, 2013).

Analysis of data obtained can be performed using one of two methods, the point analysis method where the electron probe is directed towards a single point in the specimen, or the elemental mapping method (Figure 8) where a two dimensional image reflect the level of intensity of characteristic X-rays emitted from each element (Harada and Ikuhara, 2013). The greatest disadvantage of EDX quantitative elemental analysis, however, is its low energy resolution going as low as 150 eV which could potentially lead to spectrum overlapping and misidentification of elements, therefore, caution and attention are required during analysis.

This elemental analysis technique has proven effective in many studies such as enamel demineralisation/remineralisation studies (Arnold *et al.*, 2003; Arnold *et al.*, 2006; Eggerath *et al.*, 2011; Hegde and Moany, 2012; Manoharan *et al.*, 2018), whitening studies (Souza *et al.*, 2010; Cakir *et al.*, 2011; Sathe *et al.*, 2012; Amin *et al.*, 2017; Vilhena *et al.*, 2019), and studies investigating nano-leakage at tooth\restoration interface (Hashimoto *et al.*, 2004; Yuan *et al.*,

2007; Makishi *et al.*, 2016). It was reported, however, that EDX does not have the ability to distinguish between ionic and non-ionic specimens and as a consequence it requires analysis to be performed under relative vacuum, to prevent electrons and X-rays from being absorbed by air molecules (Scimeca *et al.*, 2018). Biological samples such as dental enamel undergo dehydration before being analysed using EDX and scanning electron microscopy (SEM), to prevent the deformation of biological cells and to give realistic meaningful results. Critical point drying using a supercritical CO_2 device is usually used for that purpose (Carpentieri *et al.*, 2015). This method, however, does not prevent the loss of diffusible atoms and small molecules according to the literature; leading to the discovery of cryofixation as an alternative, to help prevent element loss during sample preparation for more accurate readings (Fernandez-Segura and Warley, 2008; Scimeca *et al.*, 2018).

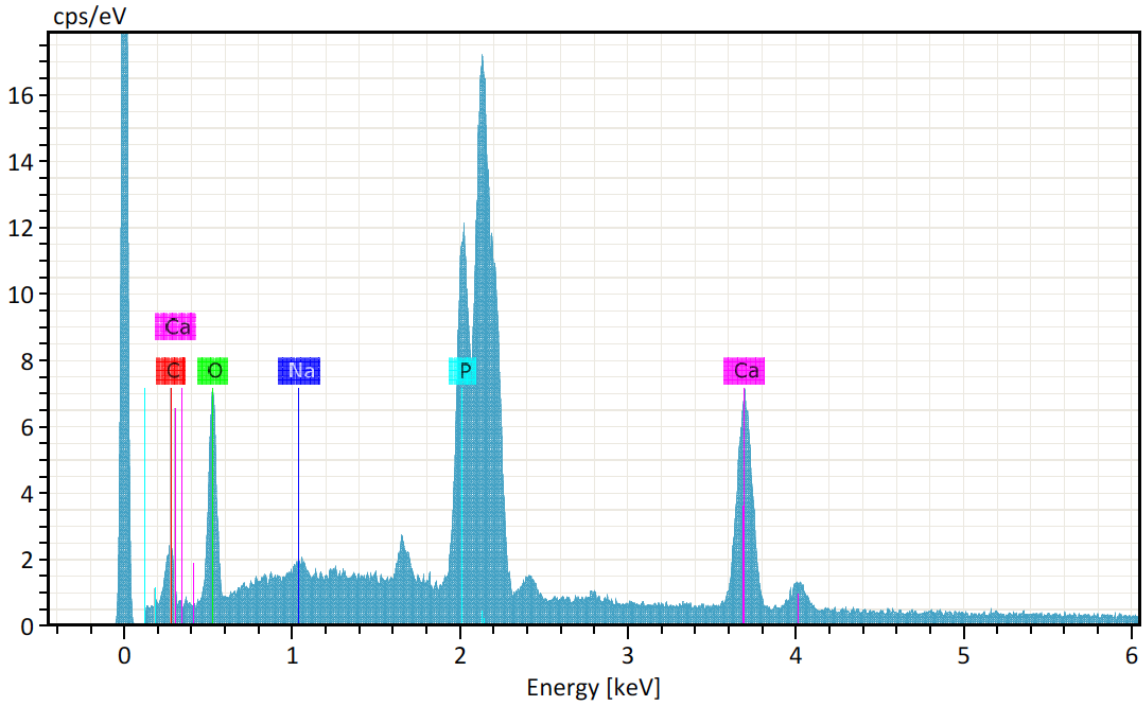


Figure 7 Shows the energy spectrum of a bovine enamel specimen. Peaks represent elements detected, and the area under each peak reflects the number of element atoms present in the area tested

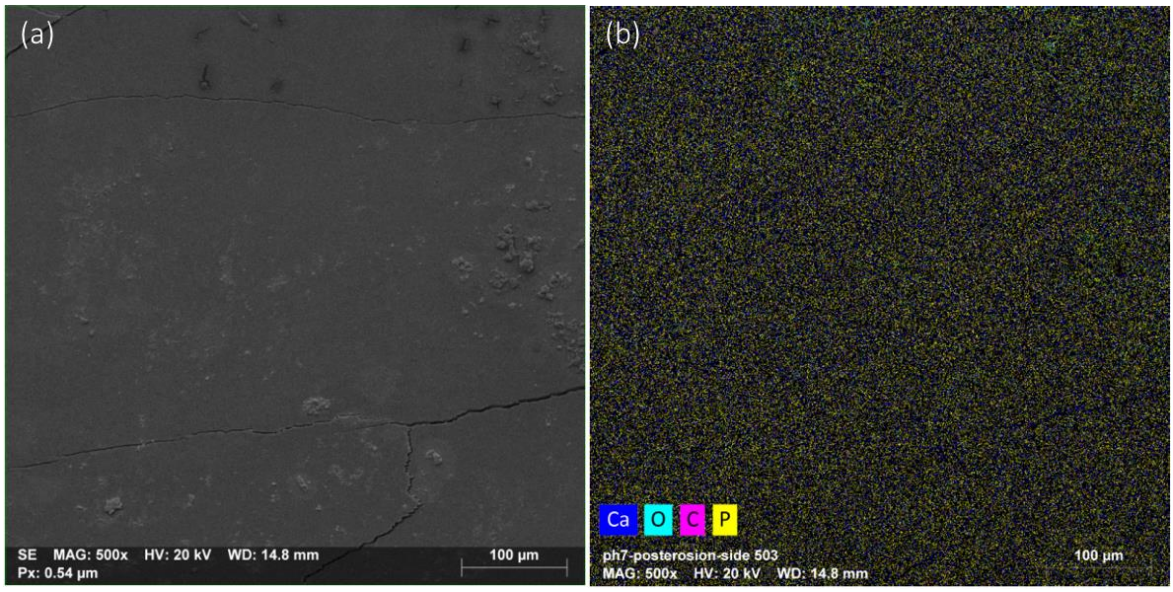


Figure 8 Shows an SEM/EDX image of bovine enamel (a) analysed through surface mapping, revealing the presence of Ca, O, C, and P across the tested surface (b).

Measuring Hardness

Hardness is defined as the material's resistance to indentation, penetration, or deformation by a harder indenter. In a micro-hardness tester, the indenter applies loads of 1N or less on the tested surface. The depth of indentation, usually less than 100 µm, is then measured using a calibrated optical microscope (Sundararajan and Roy, 2001). Micro-hardness testing is an established, non-destructive, structural characterisation method to assess phases and components of many materials such as alloys, metals, polymers, inorganic glass, etc. (Calleja and Fakirov, 2007).

Hardness can be measured by scratch, rebound, or indentation (Calleja and Fakirov, 2007; Herman, 2013). Scratch hardness is the oldest technique established and it measures the hardness of a solid by its vulnerability to getting scratched by a scale of solids (Mohs hardness scale) ranging from talc to diamond. Rebound hardness, on the other hand, involves the dynamic deformation of the tested material by dropping the indenter on its surface. The hardness value can then be calculated using the energy of impact and the size of indentation as a result. The static indentation method is the most commonly used technique to measures micro-hardness, by calculating the depth of indentation and the load applied. An example of static indentation is the Brinell hardness test, which uses a steel ball indenter that applies a static constant load on the surface tested ranging from 500-3000N for 10-30 seconds (Calleja and Fakirov, 2007; Broitman, 2017). Another example is the Knoop hardness tester which uses a rhombic-based pyramidal diamond indenter. It is considered a very popular indentation technique along with the Vickers hardness tester, which includes the use of a square pyramid diamond indenter with a 136° angle between non-adjacent faces of the pyramid. The force applied by the indenter, under a controlled rate, is held for up to 30s and the resultant indentation, down to 1µm in size, is measured using a microscopic recording device (Calleja and Fakirov, 2007). Loads applied by the Vickers hardness indenter, reach up to 10kg and in microhardness measurements, loads are usually set at 200g or less. The load applied is plotted in relation to the depth of indentation, resulting in a load-displacement curve that provides data in regards to the mechanical behavior of the material tested (Figure 9). The Vickers hardness number H_V is defined as the ratio of the applied load, *P,* to the pyramidal indentation contact area, *A* (Gong *et al.*, 1999):

$$H_V = \frac{P}{A} = \alpha P/d^2$$

Where d is the length of the diagonal impression of the indenter, and α=1.8544 for Vickers indenter.

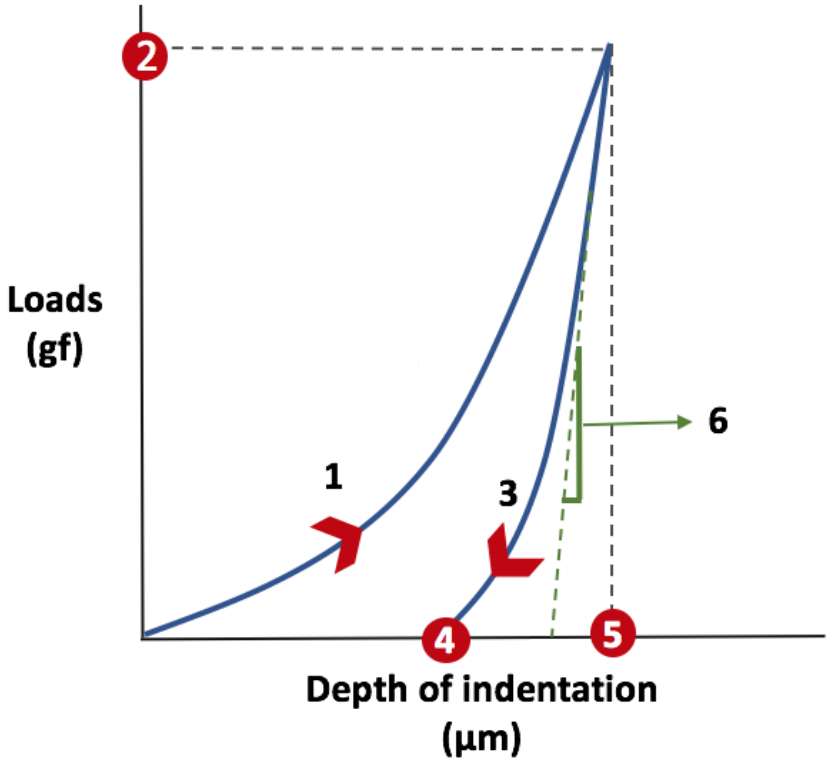


Figure 9 Shows a schematic diagram of the load-displacement curve following micro-hardness indentation. The diagram illustrates the loading of the indenter (1), the peak load (2), the unloading of the indenter (3), final indentation depth following load removal (4), maximum indentation depth (5), and the unloading stiffness.

Vickers and Knoop micro-hardness tests are associated with indentation size effect (ISE); a well-known phenomenon where the hardness number is strongly affected by the indentation size, and as the indentation size is affected by the indentation load, comparisons cannot be made between studies unless the same indentation load is used (Farges and Degout, 1989). This means that the same material will give greater hardness values under lower loads and lower hardness values under higher loads. Some attributed this phenomenon to limited resolution of the objective lens (Brown and Ineson, 1951), while others claimed that the elastic material recoil and recovery upon unloading were behind this phenomenon (Buckle, 1973). Lately, ISE was reported to be caused by the density of geometrically necessary dislocations (GNDs) which increase with the decrease of the indentation depth (Gong *et al.*, 1999; Kim *et al.*, 2017).

During micro-hardness testes, surfaces should not elastically recover after indentation, as any recoil will result in an over-estimation of hardness values by the inaccurate optical capturing of the indentation depth (Di Maio and Roberts, 2005). To prevent elastic recovery, a large indentation force is usually applied to ensure the occurrence of plastic deformation and a resultant indentation depth reflective of the material's hardness. Applying excessive indentation loads is not necessary, however, when using the reference point indentation technique (RPI) (Mallick, 2014). This indentation technique is based on a depth sensing principle using two coaxial probes. The inner probe indents the tested surface while the outer probe serves as a reference, by resting on an adjacent un-indented surface. In RPI, the depth is sensed without the need for optical recording, which allows for lower forces to be applied; making it suitable for measuring bone hardness for example in in-*vivo* studies (Gallant *et al.*, 2013).

2.9.2 Qualitative assessment

Qualitative assessment of surface changes can be achieved using two- or three-dimensional imaging systems. The most commonly used two-dimensional imaging system is the scanning electron microscope (SEM) (Figure 10). During imaging, the operator can control the focus, contrast, resolution, and magnification of the image obtained (Tafti, 2016). Conventional SEMs operate by coating specimens with an electrically conductive material such as gold. Researchers must appreciate, however, the irreversible changes caused by these coatings, and consider alternative imaging modalities such as the environmental SEM, if further investigations were planned (Field *et al.*, 2010).

Three-dimensional SEM, has the added advantage of quantitatively assessing surface topography (Tafti, 2016). It operates by taking serial 2D images of the specimen, then reconstructing these images into a 3D image (Glon *et al.*, 2014). The serial block-face imaging SEM (SBF-SEM), for instance, is a 3D-SEM imaging system that operates by sample sectioning and imaging (Denk and Horstmann, 2004; Kremer *et al.*, 2015). It starts with imaging the specimen surface, followed by sectioning and reimaging the underlying surface. The sectioning in the SBF-SEM is automatically performed using an ultramicrotome located in the SEM chamber, removing sections as thin as 20nm. Another example is the Focused Ion or Plasma Beam SEM (FIB-SEM), which operates following the same principle as the SBF-SEM, by removing very thin sections down to 5nm in thickness, by milling the external surface of the tested specimen (Kremer *et al.*, 2015).

Scanning electron microscopes have been abundantly used in dental research, such as in whitening studies (Yurdukoru *et al.*, 2003; Pinto *et al.*, 2004; Yeh *et al.*, 2005; Kemaloglu *et al.*, 2014; Vilhena *et al.*, 2019), erosion studies (Nekrashevych *et al.*, 2004; Torres *et al.*, 2010), and in demineralisation/remineralisation studies (Grewal *et al.*, 2013; Lombardini *et al.*, 2014; Colombo *et al.*, 2016). The assessment of surface quality using SEM imaging systems prove to be effective in monitoring and/or detecting early surface changes, and remain essential in various branches of research.

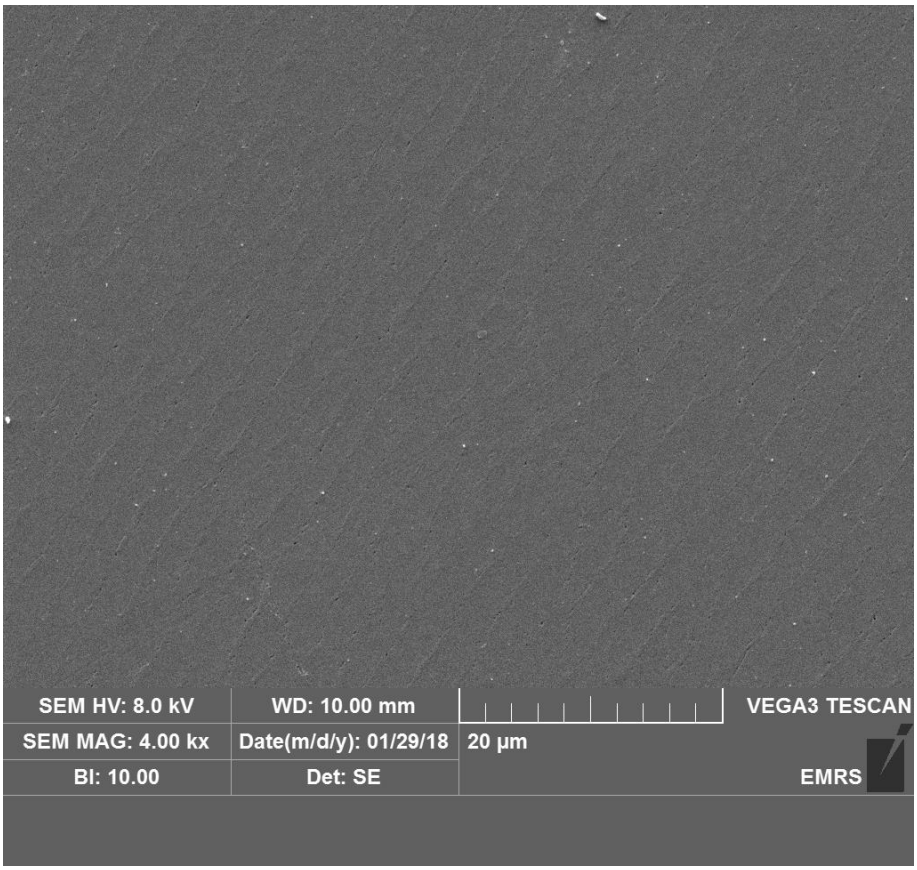


Figure 10 Shows a two dimensional SEM image of polished bovine enamel.

2.10 Statement of problem

Dental whitening is a popular, minimally invasive method to improve aesthetics and build patient confidence, however, enamel demineralisation, reduced enamel hardness, increased enamel roughness, and colour relapse are potential problems after treatment. To minimise side effects caused by whitening agents, different whitening agent concentrations and pH values were developed. Furthermore, remineralising agents such as nHA and CPP-ACP were reported to have a significant reparative capacity and the ability to restore roughness and hardness values in whitened enamel to baseline levels.

Combined effects of different HP concentrations and pH values on enamel roughness, hardness, colour change, mineral composition, and surface quality were not addressed in the literature. Studying the impact of different HP concentrations; ranging from low to high, each having an acidic, neutral, and alkaline pH value is an effective way to evaluate the concentration/pH interaction and its impact on whitened enamel. Furthermore, integrating remineralising agents such as: CPP-ACP and nHA, in the whitening protocol will help assess their ability to prevent surface damage in whitened enamel and protect treated surfaces against dietary challenges thereafter.

This study helps understand how whitening treatment options i.e. whitening agent pH, whitening agent concentration, and the application of a remineralising agent, affect the resultant whitening outcome qualitatively and quantitatively, and the susceptibility of treated enamel surfaces to dietary erosion and staining.

Chapter 3. Research questions, aims, and objectives

3.1 Research questions

1. How does HP affect enamel?
2. What are the effects of HP pH on whitened enamel?
3. What are the effects of HP concentration on whitened enamel?
4. What are the effects of remineralising agents (CPP-ACP and nHA) on whitened enamel?
5. What are the effects of dietary acids on whitened enamel?
6. What are the effects of foodstuffs/drinks known to cause extrinsic enamel discolouration on whitened enamel?

3.2 Research aims

1. Study the effects of acidic, neutral, and alkaline HP in relation to enamel roughness, hardness, colour change, mineral composition, and surface quality.
2. Investigate the effects of different HP concentrations in relation to enamel roughness, hardness, colour change, mineral composition, and surface quality.
3. Evaluate the impact of CPP-ACP and nHA remineralising pastes on whitened enamel roughness, hardness, colour change, mineral composition, and surface quality.
4. Assess the effects of dietary erosion and staining on whitened/remineralised enamel roughness, hardness, colour change, mineral composition, and surface quality.

3.3 Research objectives

Aims will be met by achieving the following objectives:

1. Developing an experimental methodology employing an established whitening protocol.
2. Developing qualitative and quantitative evaluation methods to determine enamel roughness, hardness, colour change, mineral composition, and surface quality.
3. Whitening bovine enamel using acidic, neutral and alkaline HP with low, medium, and high HP concentrations.
4. Applying remineralising agents: CPP-ACP or nHA on whitened bovine enamel after each whitening cycle.
5. Exposing whitened and/or remineralised bovine enamel to dietary staining and erosion cycles.

3.4 Programme of work

The study was structured and conducted following the sequence shown in Figure 11.

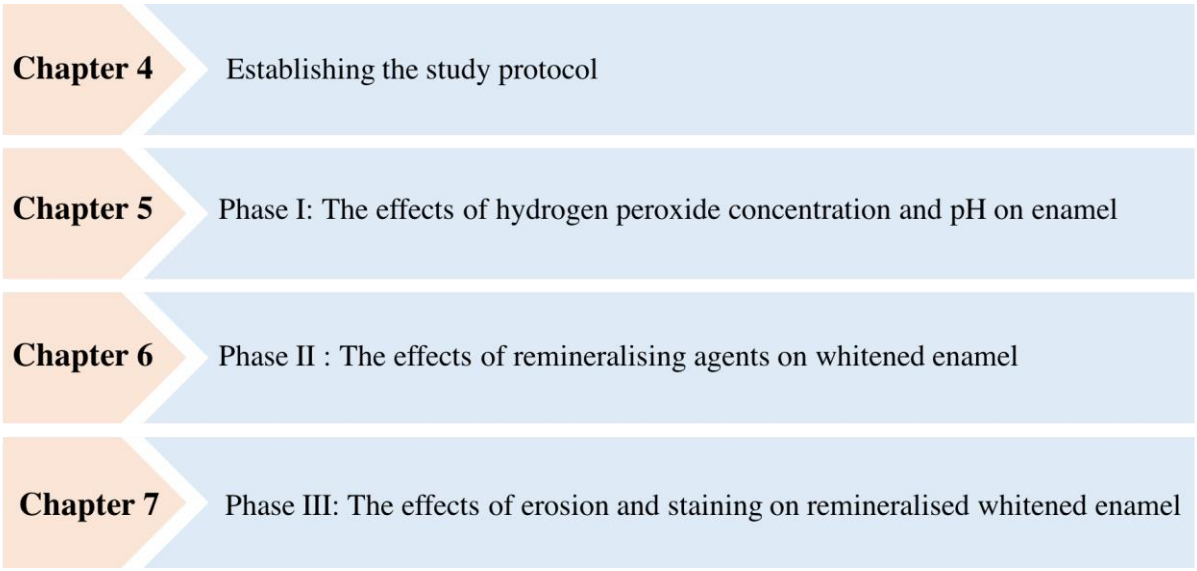


Figure 11 An illustration of study phases.

Chapter 4. Establishing the study protocol

4.1 Introduction

Preliminary studies were conducted with the purpose of developing a robust research protocol to fulfil research aims described in Chapter3. Results reported in this chapter were essential in structuring the following research phases:

Phase I: Whitening bovine enamel using acidic, neutral and alkaline HP with low, medium, and high HP concentrations. This phase was intended to investigate the impact of HP concentration and pH on bovine enamel quantitatively and qualitatively.

Phase II: Applying a remineralising agent; either CPP-ACP or nHA, on whitened bovine enamel. This phase was intended to assess the impact of remineralising agents on whitened bovine enamel quantitatively and qualitatively.

Phase III: Exposing whitened and/or remineralised bovine enamel samples to dietary staining and erosion cycles. This phase was intended to study the susceptibility of whitened/remineralised bovine enamel samples to stain uptake and erosion following exposure to dietary challenges.

4.2 Effects of solution pH and hydrogen peroxide concentration on bovine enamel

A number of experiments were undertaken to obtain solutions with different HP concentrations and pH values, monitor pH stability with time, and select a suitable test substrate. A pilot study was then conducted to examine the effects of whitening using different HP concentrations and pH values on bovine enamel, qualitatively and quantitatively.

4.2.1 Producing a range of hydrogen peroxide concentrations

Different HP concentrations were made by diluting 30 wt.% and 50 wt.% HP solutions (Sigma-Aldrich, Inc., Irvine, UK) using distilled water (DW). The resultant concentrations: 3%, 6%, 9%, 12%, 20%, and 40% (Table 2) were obtained using the following equation:

$$C_1V_1=C_2V_2$$

C_1: initial concentration (30% or 50%)
V_1: initial volume (fixed 20mL of HP)
C_2: desired concentration
V_2: final volume

Desired Concentration	Final volume	Required volume of DW
40%	50 x 20 / 40 = 25mL	25-20=5mL
20%	30 x 20 / 20 = 30mL	30-20 = 10mL
12%	30 x 20 / 12 = 50mL	50-20= 30mL
9%	30 x 20 / 9 = 66.7mL	66.7-20= 46.7mL
6%	30 x 20 / 6 = 100mL	100-20= 80mL
3%	30 x 20 / 3 = 200mL	200-20= 180mL

Table 2 Shows the required volume of distilled water to form 40%,20%, 12%, 9%, 6%, and 3% HP concentrations using the equation $C_1V_1=C_2V_2$

4.2.2 Producing a range of hydrogen peroxide pH

The pH meter (Orion 4 Star pH meter, Thermo Fisher Scientific, Leicestershire, UK) was calibrated using three standard buffer solutions with pH values of 4, 7, and 9.22 (Scientific laboratory supplies, Wilford Industrial Estate, Nottingham, UK). Three electrical potential measurements were measured (in mV) per solution. Mean values were then plotted (version 15.31, Microsoft™ Excel) against solution pH. Next, a linear regression model was fitted to the data, to generate an equation for the purpose of converting mV to pH (Figure 12).

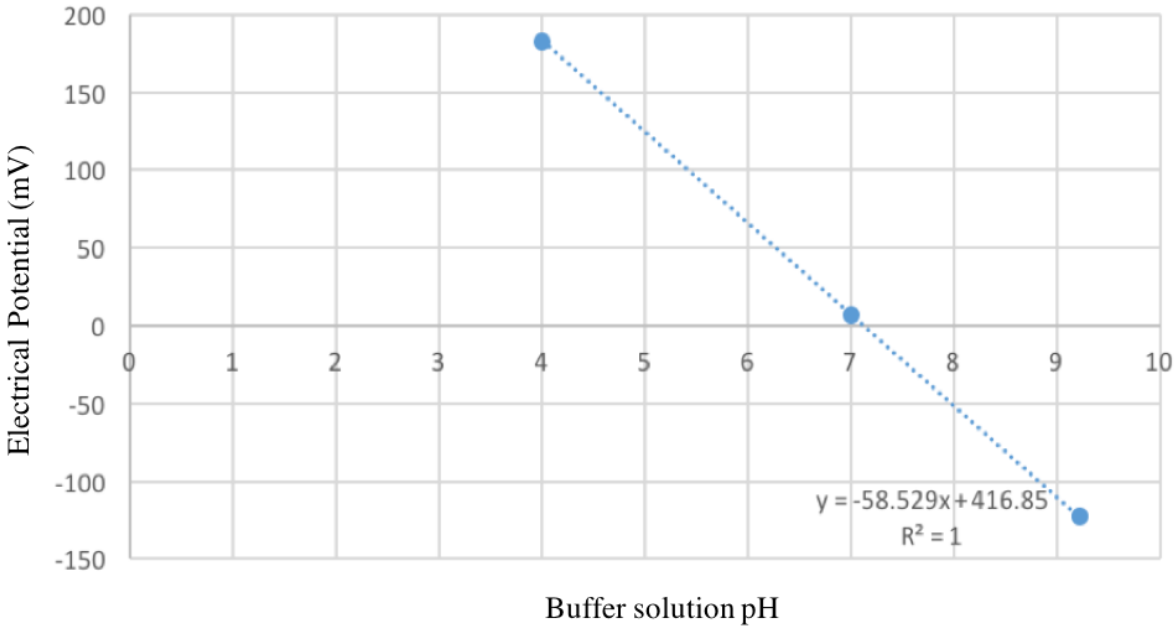


Figure 12 Shows mean electrical potential measurements in mV of three standard buffer solutions with pH values of 4, 7, and 9.22. Readings in mV were used to generate an equation to convert mV to pH.

HP was diluted using distilled water to obtain 20mL batches of each desired concentration as previously described in section 4.2.1 (Figure 13). Using a calibrated 1mL pipette, 0.25, 0.5, and 1.0M of sodium hydroxide (NaOH) and/or 0.1M of hydrochloric acid (HCl) (Sigma-Aldrich©, Inc., Irvine, UK) (Table 3) were incrementally added to each solution to adjust the HP pH and achieve acidic, neutral, and alkaline pH values of 3, 5, 7, 8, 9, and 10. Three concentrations of NaOH were used to ensure slow and controlled changes in pH were achieved. Each experiment was repeated three times per concentration, and mean volumes of NaOH and HCl needed to achieve the desired pH values were reported in Table 4.

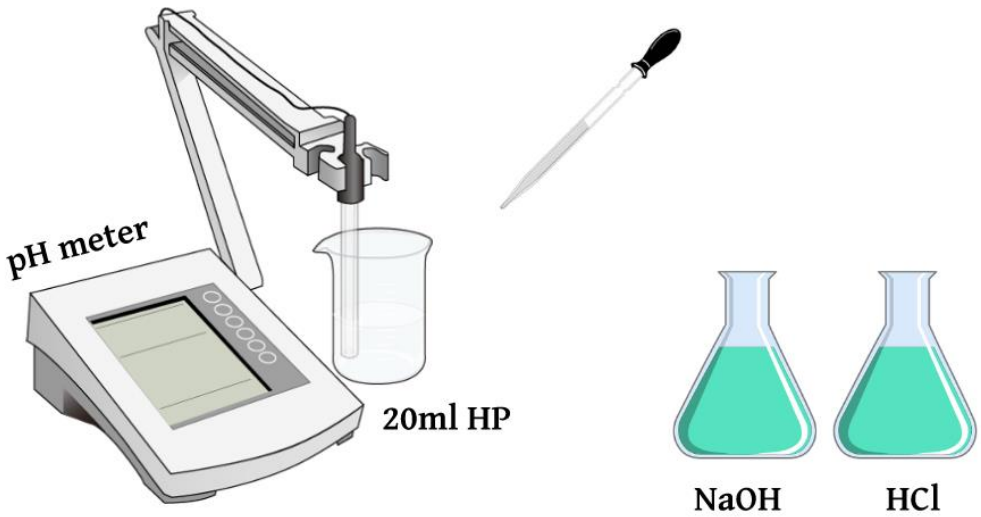


Figure 13 A diagram showing the pH adjustment of a 20mL hydrogen peroxide solution by incrementally adding NaOH and/or HCl using a 1mL pipette.

	Components	Manufacturer
0.25M NaOH	5g NaOH pellets + 500mL DW	Sigma-Aldrich©, Inc., Irvine, UK
0.5M NaOH	10g NaOH pellets + 500mL DW	
1.0M NaOH	20g NaOH pellets + 500mL DW	
0.1M HCl	4.1mL of 37% HCl + 500mL DW	

Table 3 The composition of 0.25, 0.5, and 1.0M NaOH, and 0.1M HCL solutions used to adjust HP pH values.

	Solution	Volume (ml)	pH (sol.1)	pH (sol.2)	pH (sol.3)	pH avg.	Desired pH
40%	0.5M NaOH	0.12	3.1	3.0	3.2	3.1	3
	0.5M NaOH	0.23	5.1	5.0	5.0	5.0	5
	1.0M NaOH	1.4	7.0	7.0	7.0	7.0	7
	1.0M NaOH	8.0	8.3	8.0	8.0	8.1	8
	1.0M NaOH	32.0	9.0	9.1	9.1	9.0	9
	1.0M NaOH	62.0	10.0	10.0	9.9	10.0	10
20%	-	0	3.0	3.0	3.0	3.0	3
	0.25M NaOH	0.14	5.0	5.1	5.3	5.1	5
	0.5M NaOH	0.25	7.0	6.9	7.1	7.0	7
	0.5M NaOH	1.3	8.0	8.0	8.0	8.0	8
	0.5M NaOH	6.6	9.0	8.9	9.0	9.0	9
	0.5M NaOH	20	9.9	9.8	9.8	9.9	10
12%	-	0	3.2	3.1	3.2	3.2	3
	0.25M NaOH	0.07	4.8	4.8	5.0	4.9	5
	0.25M NaOH	0.17	6.9	7.0	7.0	7.0	7
	0.5M NaOH	0.3	8.0	7.9	7.9	8.0	8
	0.5M NaOH	2.3	9.0	8.9	9.0	9.0	9
	0.5M NaOH	12	10.0	10.0	9.9	10.0	10
9%	0.1M HCl	0.25	3.0	3.0	3.0	3.0	3
	0.25M NaOH	0.06	5.2	5.2	4.9	5.1	5
	0.5M NaOH	0.05	6.8	7.0	7.0	6.9	7
	0.5M NaOH	0.16	8.0	8.1	8.0	8.0	8
	0.5M NaOH	1.0	9.0	9.0	9.0	9.0	9
	0.5M NaOH	8.0	10.0	10.0	10.0	10.0	10
6%	0.1M HCl	0.31	3.0	3.0	3.1	3.0	3
	0.25M NaOH	0.03	4.8	5.0	5.1	5.0	5
	0.25M NaOH	0.07	7.2	6.9	7.2	7.1	7
	0.5M NaOH	0.05	7.9	8.0	7.6	7.8	8
	0.5M NaOH	0.6	9.0	9.0	9.0	9.0	9
	0.5M NaOH	4.6	10.0	10.0	10.0	10.0	10
3%	0.1M HCl	0.38	3.0	3.0	3.0	3.0	3
	0.25M NaOH	0.02	5.3	5.0	4.9	5.1	5
	0.25M NaOH	0.03	7.4	7.3	7.3	7.3	7
	0.5M NaOH	0.05	8.2	8.1	8.4	8.2	8
	0.5M NaOH	0.2	8.9	8.9	9.0	9.0	9
	0.5M NaOH	1.6	10.0	9.9	10.0	10.0	10

Table 4 The required volume of 0.25, 0.5, or 1.0M NaOH and/or 0.1M HCl to achieve the desired pH values raging from 3-10 in three 20mL HP solutions (sol.) with concentrations of 40%, 20%,12%, 9%, 6%, and 3%.

4.2.3 Testing hydrogen peroxide pH stability

Time dependent pH changes in 40%, 20%, 12%, 9%, 6%, and 3% HP solutions with pH values of 3, 5, 7, 8, 9, and 10 were investigated for up to 3 hours at room temperature using a Thermo Orion 4 Star pH meter (Fisher Scientific, Leicestershire, UK). Measurements of HP pH were obtained at baseline then after 10min., 20min., 30min., 1hr, 1.5hr, 2hr, 2.5hr, and finally after 3hrs. Each HP concentration solution was divided into 6 groups of 20ml; each having a different pH value. All pH groups were then divided into 4 subgroups of 5mL solutions to obtain 4 different readings per pH at each time point (Figure 14).

Time dependent pH changes is summarised in Table 5. The solution was considered stable at pH±0.1. A rapid pH increase was recorded from T_0 to T_{3hrs} in the pH10 group at concentrations of 40% (from 10 to 11.4), 20% (from 10 to 10.9), 12% (from 10 to 10.6), 9% (from 10 to 10.8), and 6% (from 9.9 to 10.6). Moreover, a noticeable change in pH was recorded in the pH3 group with concentrations of 40% (from 3.0 to 3.5) and 20% (from 3.0 to 3.2), in addition to the pH5 group with concentrations of 9% (from 5.0 to 5.2), 6% (from 5.0 to 4.7), and 3% (from 5.0 to 5.7). Changes were also noted in the pH7 3%HP group (from 7.0 to 6.8), and in the pH8 group at concentration of 6% (from 7.9 to 7.7) and 3% (from 8.0 to 7.7). The pH9 group exhibited a rapid change in pH values at concentrations of 20% (from 9.0 to 9.4) and 3% (from 8.9 to 8.7).

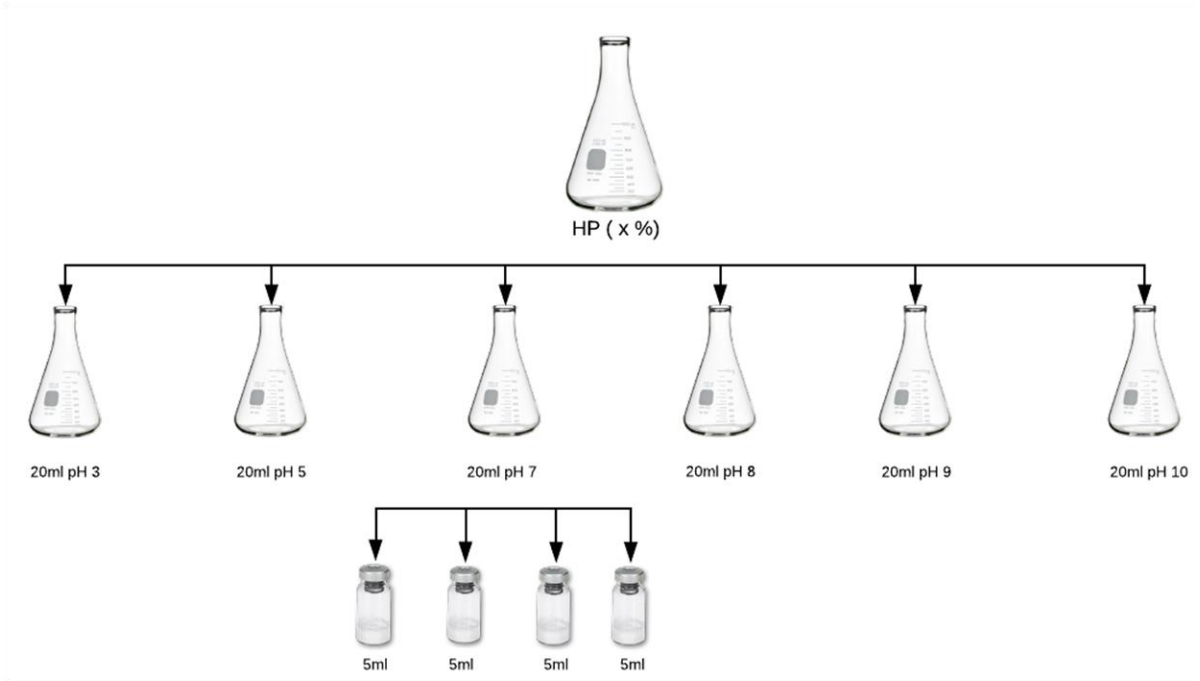


Figure 14 An Illustration of the protocol followed to assess time dependent pH changes in hydrogen peroxide solutions. Measurements of solution pH were obtained at baseline then after 10min., 20min., 30min., 1hr, 1.5hr, 2hr, 2.5hr, and finally after 3hrs.

40% HP						
T	pH 3	pH 5	pH7	pH 8	pH 9	pH 10
T_0	3.0(0.00)	5.0(0.05)	7.0(0.05)	8.0(0.00)	9.0(0.00)	10.0(0.00)
T_{10min}	3.2(0.04)	5.0(0.10)	6.9(0.05)	8.1(0.10)	9.0(0.00)	10.0(0.06)
T_{20min}	3.3(0.10)	5.0(0.05)	6.9(0.05)	8.1(0.05)	9.0(0.05)	10.0(0.10)
T_{30min}	3.4(0.10)	5.0(0.10)	6.9(0.10)	8.0(0.05)	9.0(0.00)	10.1(0.05)
T_{1hr}	3.4(0.05)	4.9(0.05)	6.9(0.10)	8.1(0.00)	9.0(0.05)	10.7(0.10)
$T_{1.5hr}$	3.4(0.06)	4.9(0.05)	6.9(0.05)	8.1(0.10)	9.0(0.08)	11.1(0.05)
T_{2hr}	3.4(0.05)	4.9(0.10)	6.9(0.05)	8.1(0.00)	9.0(0.10)	11.3(0.10)
$T_{2.5hr}$	3.5(0.06)	5.0(0.10)	6.9(0.10)	8.1(0.05)	9.0(0.05)	11.4(0.08)
T_{3hr}	3.5(0.15)	4.9(0.10)	6.9(0.05)	8.1(0.05)	9.0(0.08)	11.4(0.08)
20% HP						
T	pH 3	pH 5	pH7	pH 8	pH 9	pH 10
T_0	3.0(0.05)	5.2(0.05)	6.9(0.00)	8.0(0.00)	9.0(0.00)	10.0(0.00)
T_{10min}	3.1(0.05)	5.3(0.05)	6.9(0.00)	8.0(0.00)	9.1(0.05)	10.1(0.05)
T_{20min}	3.1(0.05)	5.3(0.00)	6.9(0.00)	8.0(0.00)	9.1(0.00)	10.2(0.05)
T_{30min}	3.1(0.05)	5.3(0.00)	6.9(0.00)	8.0(0.00)	9.1(0.00)	10.3(0.05)
T_{1hr}	3.1(0.09)	5.3(0.05)	6.9(0.00)	8.0(0.00)	9.1(0.05)	10.5(0.06)
$T_{1.5hr}$	3.1(0.08)	5.3(0.05)	6.9(0.05)	7.9(0.00)	9.1(0.00)	10.6(0.08)
T_{2hr}	3.1(0.08)	5.3(0.05)	6.9(0.05)	7.9(0.05)	9.1(0.05)	10.7(0.08)
$T_{2.5hr}$	3.1(0.13)	5.3(0.05)	6.9(0.05)	8.0(0.05)	9.4(0.05)	10.8(0.08)
T_{3hr}	3.2(0.10)	5.3(0.05)	6.9(0.06)	7.9(0.05)	9.4(0.08)	10.9(0.08)
12% HP						
T	pH 3	pH 5	pH 7	pH 8	pH 9	pH 10
T_0	3.0(0.00)	5.2(0.05)	7.0(0.00)	8.0(0.00)	9.0(0.00)	10.0(0.00)
T_{10min}	3.0(0.00)	5.1(0.10)	6.9(0.05)	8.0(0.00)	9.0(0.05)	10.1(0.00)
T_{20min}	3.0(0.00)	5.1(0.10)	6.9(0.00)	7.9(0.05)	9.0(0.00)	10.2(0.05)
T_{30min}	3.0(0.00)	5.2(0.10)	6.9(0.00)	7.9(0.00)	9.0(0.00)	10.2(0.00)
T_{1hr}	3.0(0.05)	5.2(0.20)	6.9(0.00)	7.9(0.00)	9.0(0.00)	10.3(0.00)
$T_{1.5hr}$	3.0(0.05)	5.2(0.13)	6.9(0.05)	7.9(0.00)	9.0(0.00)	10.4(0.05)
T_{2hr}	3.0(0.05)	5.2(0.20)	6.9(0.05)	7.9(0.00)	9.0(0.00)	10.5(0.05)
$T_{2.5hr}$	3.0(0.10)	5.2(0.24)	6.9(0.05)	7.9(0.00)	9.0(0.00)	10.6(0.05)
T_{3hr}	3.0(0.10)	5.2(0.30)	6.9(0.10)	7.9(0.00)	9.0(0.00)	10.6(0.10)
9% HP						
T	pH 3	pH 5	pH 7	pH 8	pH 9	pH 10
T_0	2.9(0.00)	5.0(0.05)	6.9(0.00)	8.0(0.00)	9.0(0.00)	10.0(0.00)
T_{10min}	2.7(0.05)	5.0(0.05)	6.9(0.08)	8.0(0.00)	9.0(0.00)	10.1(0.00)
T_{20min}	2.9(0.00)	5.0(0.10)	6.9(0.06)	7.9(0.05)	9.0(0.00)	10.3(0.00)
T_{30min}	2.9(0.05)	5.0(0.13)	6.9(0.06)	7.9(0.05)	9.0(0.00)	10.4(0.06)
T_{1hr}	2.9(0.05)	5.0(0.10)	6.8(0.10)	7.9(0.05)	9.0(0.00)	10.5(0.05)
$T_{1.5hr}$	2.9(0.00)	5.1(0.08)	6.8(0.10)	7.9(0.00)	9.0(0.00)	10.6(0.06)
T_{2hr}	2.9(0.00)	5.0(0.06)	6.8(0.10)	7.9(0.00)	9.0(0.00)	10.7(0.06)
$T_{2.5hr}$	2.8(0.05)	5.2(0.24)	6.8(0.14)	7.9(0.00)	9.0(0.00)	10.7(0.05)
T_{3hr}	2.9(0.00)	5.2(0.20)	6.8(0.15)	7.9(0.05)	9.0(0.00)	10.8(0.10)
6% HP						
T	pH 3	pH 5	pH 7	pH 8	pH 9	pH 10
T_0	2.8(0.05)	5.0(0.05)	6.9(0.05)	7.9(0.00)	9.0(0.00)	9.9(0.00)
T_{10min}	2.9(0.00)	5.1(0.05)	7.0(0.06)	7.9(0.00)	9.0(0.06)	10.0(0.00)
T_{20min}	2.9(0.00)	5.1(0.05)	6.9(0.05)	7.9(0.00)	8.9(0.00)	10.0(0.05)
T_{30min}	2.9(0.05)	5.0(0.20)	6.9(0.00)	7.9(0.05)	8.9(0.00)	10.1(0.00)
T_{1hr}	2.9(0.00)	4.9(0.05)	6.9(0.00)	7.8(0.00)	8.9(0.00)	10.2(0.05)
$T_{1.5hr}$	2.9(0.05)	4.9(0.06)	6.9(0.00)	7.8(0.00)	8.9(0.00)	10.4(0.05)
T_{2hr}	2.9(0.13)	4.9(0.06)	6.9(0.00)	7.8(0.00)	8.9(0.00)	10.5(0.05)
$T_{2.5hr}$	3.0(0.20)	4.8(0.00)	6.9(0.05)	7.8(0.05)	8.9(0.00)	10.5(0.10)
T_{3hr}	2.9(0.13)	4.7(0.05)	6.9(0.05)	7.7(0.05)	8.9(0.05)	10.6(0.05)
3% HP						
T	pH 3	pH 5	pH 7	pH 8	pH 9	pH 10
T_0	3.0(0.00)	5.0(0.05)	7.0(0.00)	8.0(0.10)	8.9(0.05)	9.9(0.00)
T_{10min}	3.0(0.00)	5.3(0.34)	6.9(0.05)	8.0(0.10)	8.9(0.00)	9.9(0.00)
T_{20min}	3.0(0.00)	5.4(0.30)	6.9(0.05)	8.0(0.10)	8.9(0.00)	9.9(0.00)
T_{30min}	3.0(0.00)	5.4(0.26)	6.8(0.10)	8.0(0.10)	8.9(0.00)	9.9(0.00)
T_{1hr}	3.0(0.00)	5.5(0.25)	6.8(0.05)	7.9(0.10)	8.9(0.00)	9.9(0.00)
$T_{1.5hr}$	3.0(0.00)	5.5(0.26)	6.8(0.10)	7.8(0.05)	8.8(0.00)	9.9(0.00)
T_{2hr}	3.0(0.05)	5.6(0.26)	6.9(0.10)	7.8(0.10)	8.8(0.00)	9.9(0.00)
$T_{2.5hr}$	3.0(0.05)	5.6(0.30)	6.8(0.10)	7.8(0.10)	8.8(0.06)	9.9(0.05)
T_{3hr}	3.1(0.05)	5.7(0.30)	6.8(0.08)	7.7(0.08)	8.7(0.00)	9.9(0.05)

Table 5 Time dependent pH changes in HP solutions with concentrations of 40%, 20%, 12%, 9%, 6%, and 3% and pH values of 3, 5, 7, 8, 9, and 10. Measurements were taken using a pH meter at baseline, after 10 min., 20 min., 30 min., 1hr.,1.5hr., 2hr., 2.5 hr., and 3hr. Values in parentheses are standard deviations.

4.2.4 Test substrate

Pre-sectioned and lapped (6×6mm) bovine enamel blocks (Intertek group© plc., Cheshire, UK) were selected as test substrates, Figure 15. According to the manufacturer, after extraction, crowns were separated from roots. Each crown was then cut and trimmed to the desired sample size and shape using a hard tissue saw and a Silfradent 801trimmer (Silfradent©, Italy). Samples were polished in two stages; starting with a PM5 lapping and polishing machine (Logitech© Limited, UK) using a 9µm aluminum oxide slurry, followed by a grinder/polisher (Saphir550, ATM GmbH©, Germany) using a 1µm aluminium oxide slurry then a 0.04µm colloidal silica suspension.

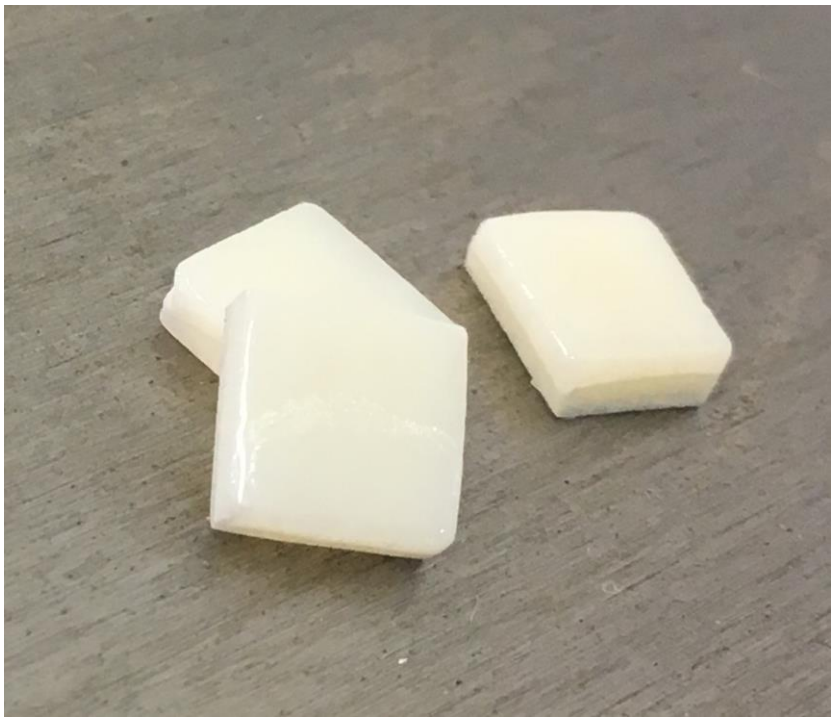


Figure 15 Bovine enamel blocks (6×6mm).

Polished and unpolished bovine enamel samples were compared quantitatively and qualitatively. Enamel roughness average values (Ra) in 20 bovine samples were assessed using a diamond cone tip stylus profilometer *(Mitutoyo Surftest SV-2000 Mitutoyo, Halifax, UK)* with a dedicated analysis software (Surfpak- SV V1.600) (Figure 16). The stylus was held at 90° to each sample surface with an assessment length of 4mm at three separate but parallel areas per sample, with a contact force of 4mN and tip radius of 5µm.

Figure 16 A diamond cone tip stylus profilometer (Mitutoyo Surftest SV-2000 Mitutoyo, Halifax, UK) (a) measuring a bovine enamel sample (b).

Qualitative analysis of surface features was performed using SEM (Tescan Vega SEM 3LMU, Tescan, Cambridge) on 3 polished and 3 unpolished bovine enamel samples (Figure 17). Enamel samples were prepared for SEM imaging as follows:

1- Enamel samples were fixed in 2% glutaraldehyde in Sorenson's phosphate buffer for 24 hours.
2- Samples were then rinsed and gradually dehydrated using 25% ethanol, 50% ethanol, 75% ethanol, and finally 100% ethanol (Fisher Scientific©, Leicestershire, UK).
3- Samples were then critically point dried with carbon dioxide using a beltic critical point dryer (Leica Geosystems Ltd, Tongwell, Milton Keynes).
4- Samples were covered with a 5-10nm gold coating, using a Polaron SEM coating unit (Polaron E5000 SEM Coating unit, Quorum Technologies Ltd, East Sussex).
5- Finally, samples were mounted on aluminium stubs, ready for imaging.

Images were captured at three magnifications (×300, ×1000, and ×4000) to gain both a general overview and an in-depth characterisation of enamel microstructure.

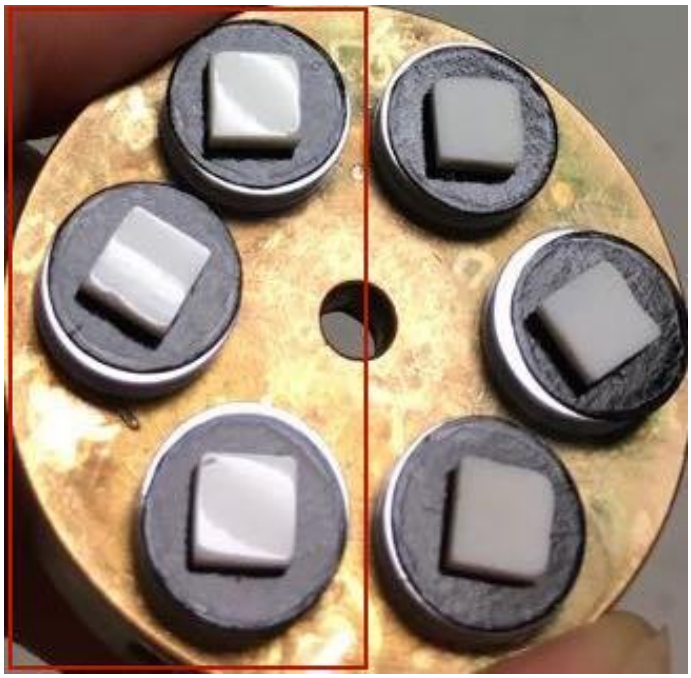


Figure 17 Mounted bovine enamel samples. Samples in the red box have a 0.04 µm polish, and the other three samples are unpolished.

Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics version 24.0 software. Roughness average values of polished and unpolished bovine enamel samples were not normally distributed ($P < 0.050$, Shapiro-Wilk) with no equality of variance ($P < 0.050$, Brown-Forsythe) and therefore, data were analysed using the non-parametric Mann-Whitney U test. Values were displayed using box and whisker plots for a visual representation of the differences between groups.

Results

Enamel roughness (Ra)

Average Ra values in polished and unpolished bovine enamel samples are presented in Table 6. In comparison to polished bovine samples, the unpolished group showed less variability in recorded Ra values. On the other hand, the majority of polished bovine samples had lower average Ra values and an evident surface gloss. These differences, however, were not statistically significant (P=0.075) (Figure 18).

Sample No.	**Group 1 0.04µ*m* polish**	**Group 2 unpolished**
1	0.26 (0.15)	0.28 (0.10)
2	0.17 (0.13)	0.23 (0.04)
3	0.25 (0.11)	0.24 (0.04)
4	0.47 (0.23)	0.22 (0.01)
5	0.04 (0.01)	0.24 (0.04)
6	0.15 (0.07)	0.25 (0.06)
7	0.21 (0.12)	0.27 (0.03)
8	0.16 (0.00)	0.21 (0.02)
9	0.13 (0.06)	0.23 (0.01)
10	0.06 (0.02)	0.23 (0.01)
Range	0.04-0.47µ*m*	0.21-0.28µ*m*

Table 6 Roughness average values (Ra) for 10 polished and 10 unpolished bovine enamel samples. Each figure is an average value of three independent readings per sample. Values between parenthesis are standard deviations.

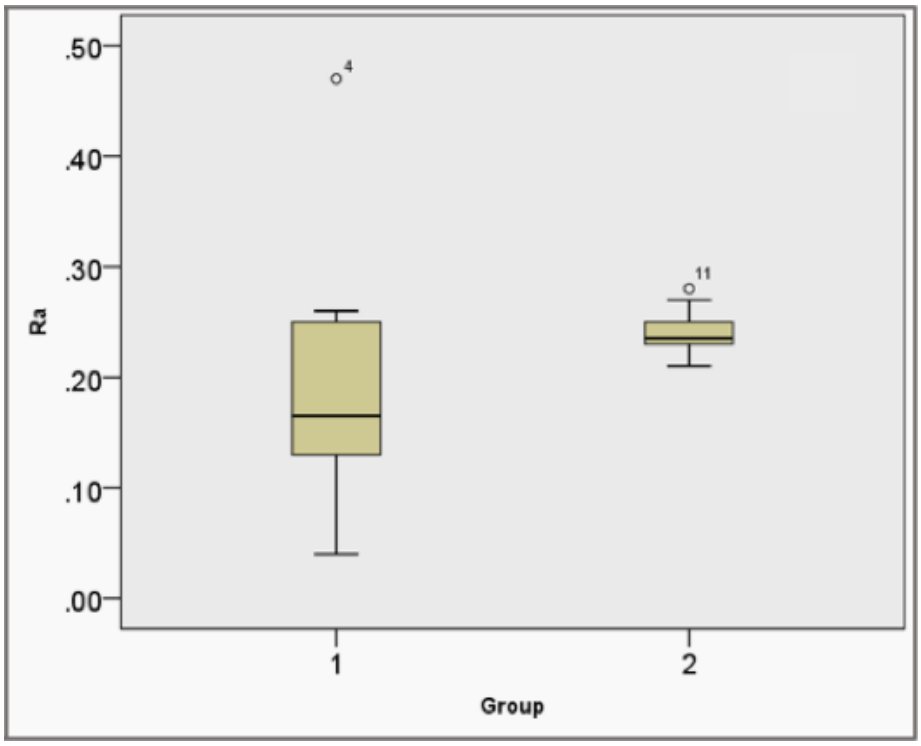


Figure 18 Box and whiskers plot of Ra values in polished (1) and unpolished (2) bovine enamel groups. Error bars represent the variability of data.

Enamel surface quality

Scanning Electron Microscopy images using Tescan Vega 3LMU SEM (Tescan Vega SEM LMU, Tescan, Cambridge) of polished and unpolished bovine enamel samples are shown in Figure 19. Images were captured at 300x, 1000x, and 4000x magnification. Enamel cracks were observed in both groups, possibly attributed to the sectioning, lapping, transport, and the critical point drying required for SEM imaging (Xu *et al.*, 1997). The unpolished enamel showed greater surface irregularities with visible scratches under 1000x magnification.

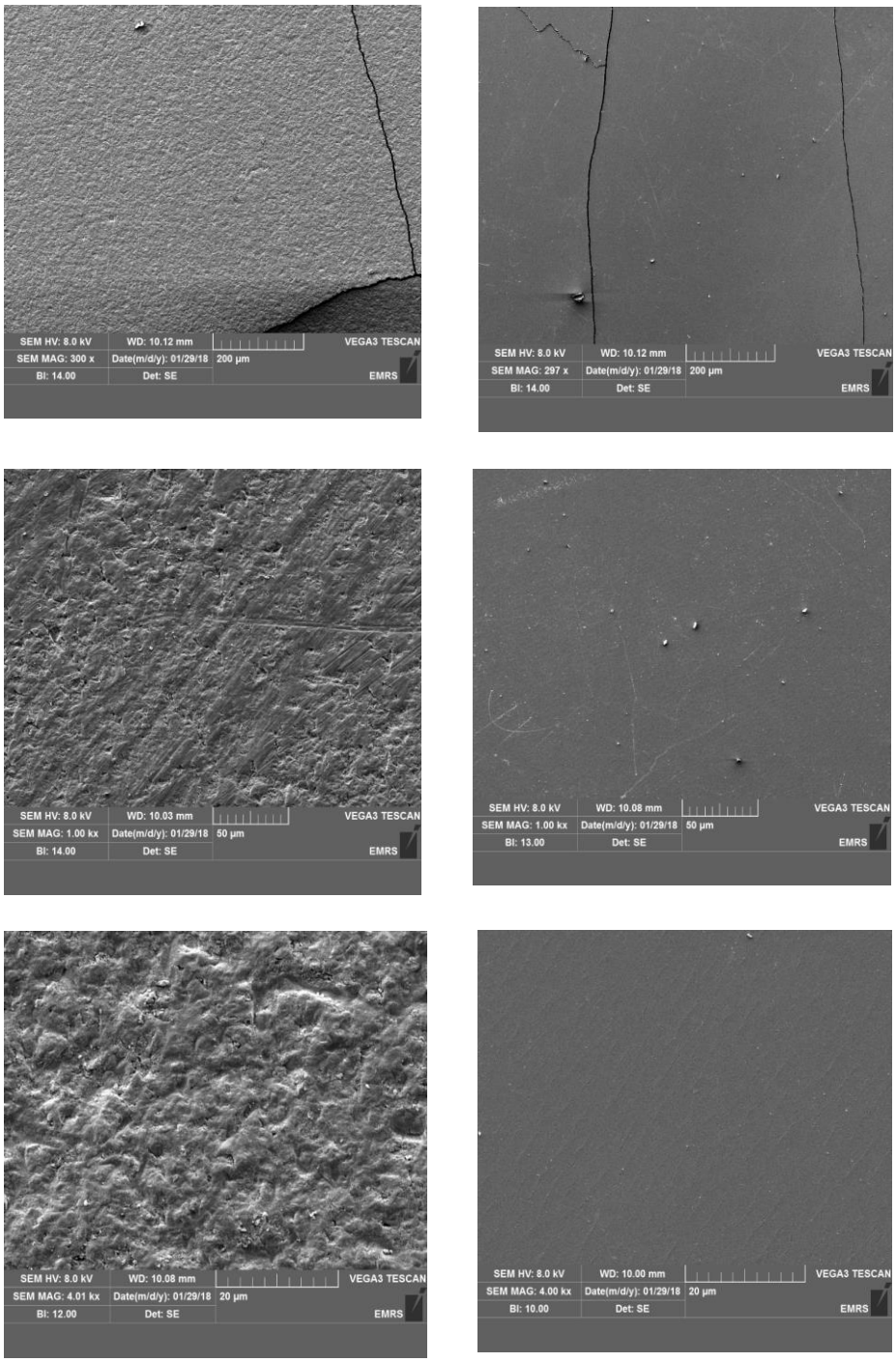


Figure 19 Scanning Electron Microscopy images of unpolished bovine enamel samples (left column) and polished bovine enamel samples (right column) under x300, x1000, and x4000 magnification. Note the smooth enamel surface in the polished group as compared to the unpolished surfaces.

4.2.5 Assessing the effects of hydrogen peroxide concentration and pH, and whitening duration on bovine enamel

Introduction

A pilot study was conducted to assess the impact of exposure to HP at different concentrations and pH values on polished bovine enamel samples.

Materials and Methods

The study involved 35 polished bovine enamel samples, treated using 6 wt.% and 12 wt.% HP with pH values of 5, 7, and 9 (n=5 per pH) prepared as described in sections 4.2.1 and 4.2.2. Samples were treated for 2 hours, daily, for 14 days. The control group (C) (n=5) was treated with Dulbecco's phosphate buffered saline (PBS) (Gibco, Paisley, UK) (pH 7.4). Samples were stored in 1% chloramine-T (Fisher Scientific©, Leicestershire, UK) after each whitening treatment at 6°C.

Enamel roughness measurements were obtained at baseline (BL), day7, and day14. Three Ra measurements were obtained per sample using a diamond cone tip stylus profilometer (Mitutoyo Surftest SV-2000 Mitutoyo, Halifax, UK) with a dedicated analysis software (Surfpak- SV V1.600).

Colour measurements were taken using a spectrophotometer with a 4mm aperture (Ci62, X-Rite Europe GmbH, Regensdorf, Switzerland) (Figure 20) at baseline, day 7, and day 14. Following the calibration of the device according to manufacturer's instructions, colour measurements were obtained using the CIE Lab system with a CIE standard illumination setting (D65/10°) corresponding to midday average light, viewed at a standard 10° angle. Three independent readings were taken for each sample against a white background. To create a flat surface necessary for colour measurement, a customised clear acrylic sheet was used. Samples were placed in a 6x6mm square opening created in the acrylic sheet; corresponding to the size of enamel samples tested. Overall colour change (ΔE) was calculated using the following equation:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2}$$

Where L^*_2, a^*_2, and b^*_2 are colour coordinates after treatment, while L^*_1, a^*_1, and b^*_1 are colour coordinates at baseline.

Figure 20 The spectrophotometer used to measure colour changes at baseline, day 7, and day 14 of the whitening cycle.

Quantitative evaluation of mineral content in treated enamel samples (n=2 per pH) was undertaken using energy dispersive x-ray spectroscopy (EDX) set at 10 kV accelerating voltage (Bruker Xflash 6130, Bruker®, Camarillo, CA) and *qualitative assessment* of enamel microstructure was performed using Tescan Vega 3LMU SEM (Tescan Vega SEM LMU, Tescan, Cambridge). Samples were prepared for EDX and SEM as described in Section 4.2.4. Images were firstly obtained using SEM, and processed using its relevant TESCAN software under low, medium, and high magnifications ranging from 30x to 5000x. After which surface mapping was performed using EDX and mineral content was generated using Quantax Esprit 2.1 software (Bruker®, Camarillo, CA).

Study design

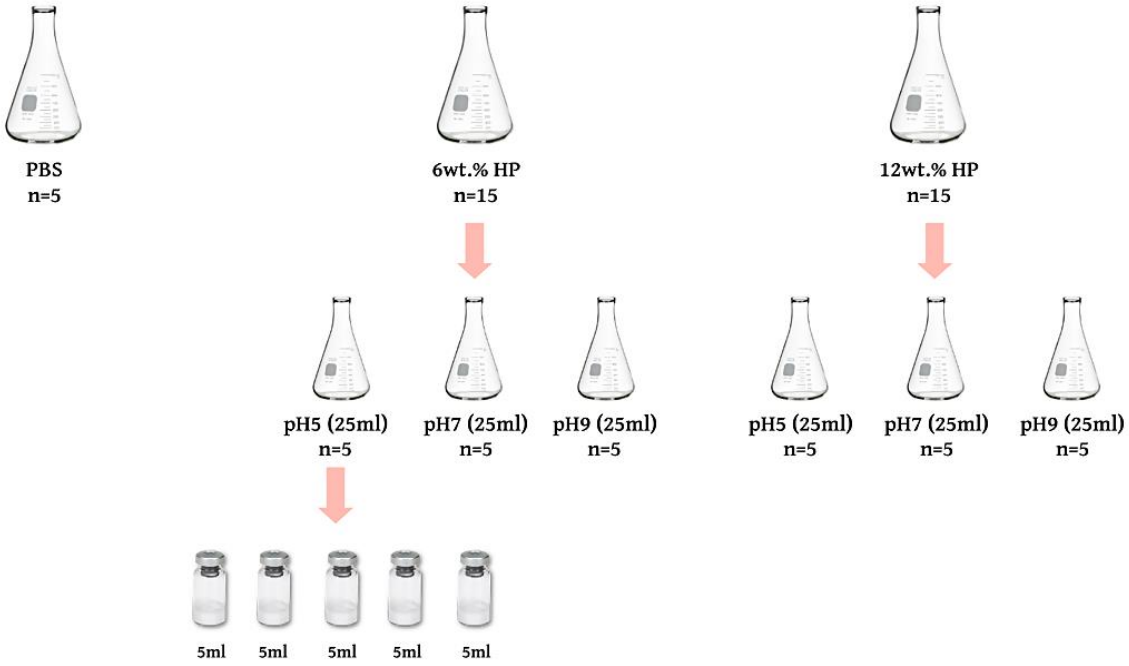


Figure 21 An illustration of the pilot study conducted using two HP concentrations of 6% and 12% with pH values of 5, 7, and 9. After adjusting HP pH, the solution was divided into five 5mL solutions placed in 5 separate vials; each containing a bovine enamel sample. The control group was treated with phosphate buffer saline during each whitening cycle.

Statistical analysis

Statistical analysis regarding the effects of whitening duration, HP concentration, and HP pH on enamel roughness, colour, and mineral composition was performed using SigmaPlot for Windows version 13.0, Build 13.0.0.83 (Systat Software 2014®). Normality tests were performed using Shapiro-Wilk, and equal variance was tested using Brown-Forsythe Three-way Analysis of Variance (ANOVA) was used to test the effects of independent variables: time (BL, day 7, and day 14), HP concentration (C, 6% and 12%), and HP pH (C, 5, 7, and 9). All pairwise multiple comparisons were performed using Holm-Sidak method with an overall significance of 0.05.

Results

Enamel roughness (Ra)

Average Ra values of treated enamel surfaces at BL, day7, and day14 are shown in Table 7. There are no statistically significant changes in average Ra values by the effect of time, HP pH, or HP concentration (P>0.05). In addition, interactions between time, concentration, and pH showed no statistically significant differences in average Ra values.

	12%			6%			Control
	pH5	pH7	pH9	pH5	pH7	pH9	
BL	0.07 (0.01)	0.07 (0.03)	0.07 (0.03)	0.07 (0.01)	0.07 (0.05)	0.04 (0.01)	0.09 (0.04)
Day7	0.06 (0.04)	0.09 (0.06)	0.09 (0.03)	0.07 (0.05)	0.06 (0.02)	0.06 (0.04)	0.05 (0.01)
Day14	0.10 (0.11)	0.07 (0.05)	0.07 (0.03)	0.07 (0.04)	0.04 (0.01)	0.09 (0.08)	0.11 (0.09)

Table 7 Average Ra measurements of treated bovine enamel (n=35) at BL, day 7, and day 14. Values between parenthesis are standard deviations.

Enamel colour

Whitening duration and HP concentration significantly affected all colour parameters in whitened enamel samples in comparison to the control group ($P \leq 0.05$, Table 8). Enamel treated using pH5 12%HP had the greatest ΔE value (ΔE=10.39), followed by pH9 12%HP (ΔE=9.63), pH5 6%HP (ΔE=9.41), pH7 12%HP (ΔE=9.26), pH9 6%HP (ΔE=9.14), pH7 6%HP (ΔE=8.71), and least ΔE values were recorded in the control group (ΔE= 2.61). Statistically significant changes in colour parameters L*, a*, b* and ΔE were recorded in whitened enamel samples from BL to day7 and from BL to day14. Statistically significant changes in colour parameters L*, b* and ΔE were recorded on whitened enamel samples from day7 to day14. The pH5 6% HP solution caused a significant change in L*, a*, b* and ΔE values from day7 to day14. The 12% HP solution produced significantly greater L* values than those produced by the 6% HP solution. Within each concentration group, colour changes were not significantly different between acidic, neutral, and alkaline HP ($P > 0.05$).

PH	Measuring Time	L*	a*	b*	ΔE		
			12%				
PH5	BL	80.70[a] (1.30)	-0.34[a] (0.21)	9.20[a] (1.76)	9.1		10.39
	Day7	87.52[b] (0.49)	-1.42[b] (0.16)	3.36[b] (0.95)		1.29	
	Day14	88.20[c] (0.29)	-1.38[b] (0.15)	2.33[c] (1.0)			
PH7	BL	80.93[a] (0.69)	-0.70[a] (0.20)	8.10[a] (0.79)	8.2		9.26
	Day7	87.16[b] (1.23)	-1.45[b] (0.10)	2.90[b] (0.68)		1.06	
	Day14	87.73[c] (1.39)	-1.44[b] (0.10)	2.03[c] (0.71)			
PH9	BL	80.81[a] (0.87)	-0.67[a] (0.31)	9.00[a] (1.34)	8.4		9.63
	Day7	86.64[b] (0.45)	-1.52[b] (0.17)	3.11[b] (1.09)		1.23	
	Day14	87.59[c] (0.59)	-1.48[b] (0.15)	2.43[c] (0.95)			
			6%				
PH5	BL	80.43[a] (0.69)	-0.61[a] (0.21)	8.83[a] (1.37)	8.0		9.41
	Day7	85.70[b] (0.67)	-1.52[b] (0.19)	2.87[b] (1.05)		1.41	
	Day14	86.80[c] (0.75)	-1.48[c] (0.16)	2.05[c] (1.04)			
PH7	BL	81.37[a] (1.27)	-0.60[a] (0.14)	8.61[a] (0.98)	7.3		8.71
	Day7	85.56[b] (0.66)	-1.53[b] (0.09)	2.85[b] (0.29)		1.41	
	Day14	86.63[c] (1.03)	-1.51[b] (0.11)	2.02[c] (0.36)			
PH9	BL	80.11[a] (1.32)	-0.55[a] (0.44)	8.80[a] (1.56)	7.8		9.14
	Day7	85.42[b] (1.07)	-1.46[b] (0.10)	3.17[b] (1.42)		1.34	
	Day14	86.43[c] (0.82)	-1.46[b] (0.08)	2.31[c] (1.20)			
			Control				
PBS pH7.4	BL	81.24[a] (0.74)	-0.50[a] (0.18)	8.40[a] (1.55)	1.6		2.61
	Day7	81.36[a] (0.73)	-0.61[a] (0.28)	8.93[a] (2.0)		1.01	
	Day14	82.06[b] (0.96)	-0.43[b] (0.29)	8.41[b] (1.8)			

Table 8 Average colour measurements of treated bovine enamel (n=35) at BL, day 7, and day 14. ΔE was measured from BL to day 7 (green), from BL to day 14 (blue), and from day 7 to day 14 (grey). Values between parenthesis are standard deviations and different superscript letters indicate a statistically significant difference within each pH group at different time points.

Mineral composition

There were no statistically significant differences in mean atomic % values of calcium (Ca), phosphorus (P), and carbon (C) between treatment groups (P>0.05) (Table 9).

Element	Control	6% HP			12% HP		
		pH5	pH7	pH9	pH5	pH7	pH9
Ca	36.8(1.3)	37.4(0.3)	36.0(0.8)	37.5(0.3)	37.4(0.3)	37.7(1.5)	39.0(0.3)
P	1.3(0.1)	1.4(0.0)	1.3(0.0)	1.3(0.1)	1.4(0.0)	1.4(0.0)	1.4(0.1)
C	5.1(1.5)	6.7(0.6)	5.6(0.2)	7.1(2.0)	5.9(0.1)	5.1(1.0)	4.8(0.5)

Table 9 Mean atomic % of each element relative to the total number of atoms present. There are no statistically significant differences in mean atomic % values between all groups. Values between parenthesis are standard deviations.

Surface quality

Scanning electron microscopy images of treated bovine enamel samples are shown under 200x, 2000x, and 5000x magnification (Figure 22-24). There appears to be a difference in surface quality between the control group and whitened enamel groups. Under low magnification, enamel cracks were visible across all treated enamel surfaces. Under 2000x magnification, on the other hand, enamel prisms were exposed in whitened enamel surfaces; appearing as perikymata-like lines. The exposure of enamel prisms was more pronounced under high magnification in whitened enamel samples as compared to the control group. Under all magnifications, enamel whitened using 6%HP and 12%HP showed similar surface changes.

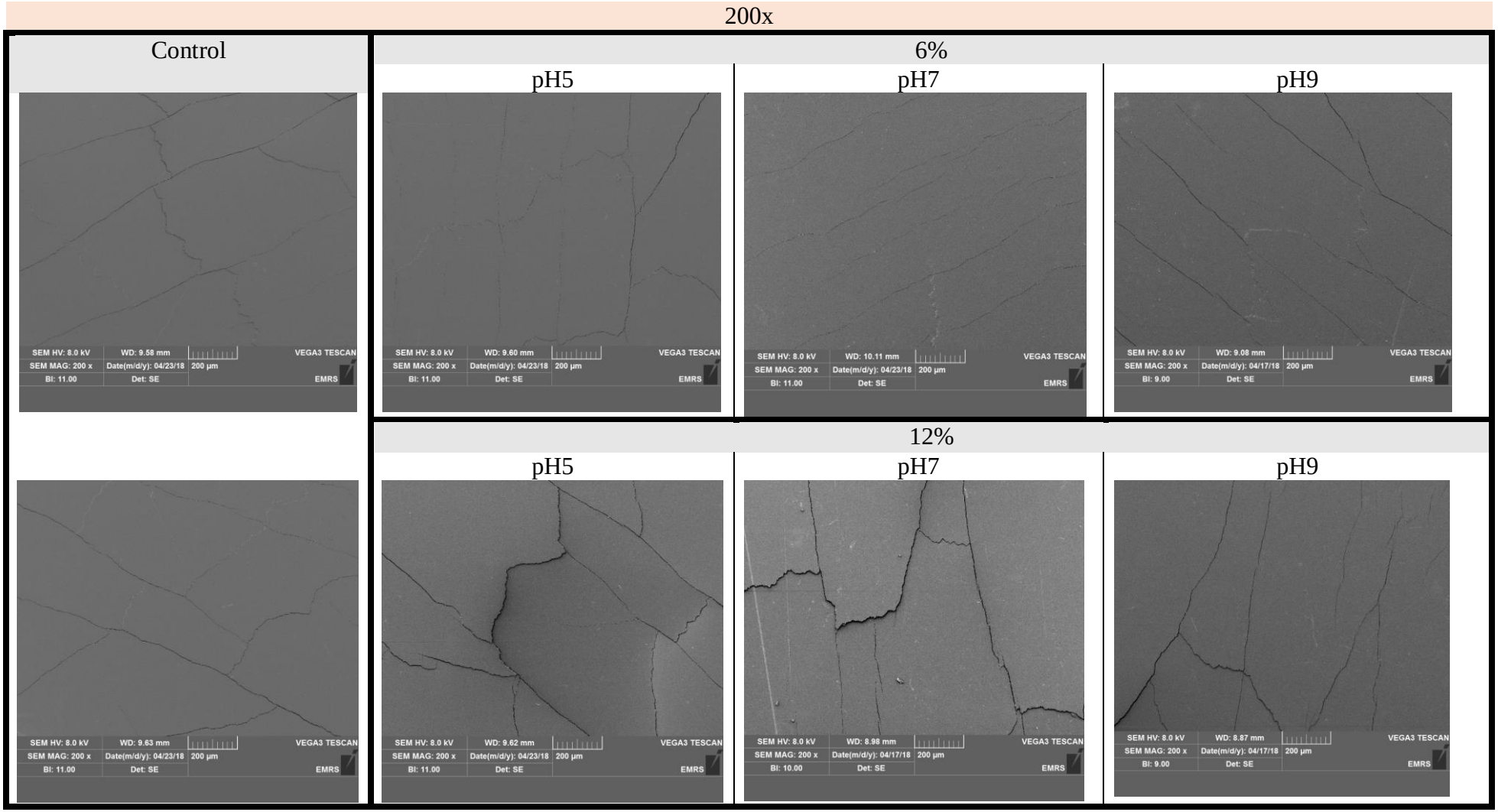


Figure 22 Scanning electron microscopy images of the control (left column), 6%HP (top row), and 12%HP (bottom row) groups under 200x magnification. Enamel cracks are visible across all treated surfaces under low magnification.

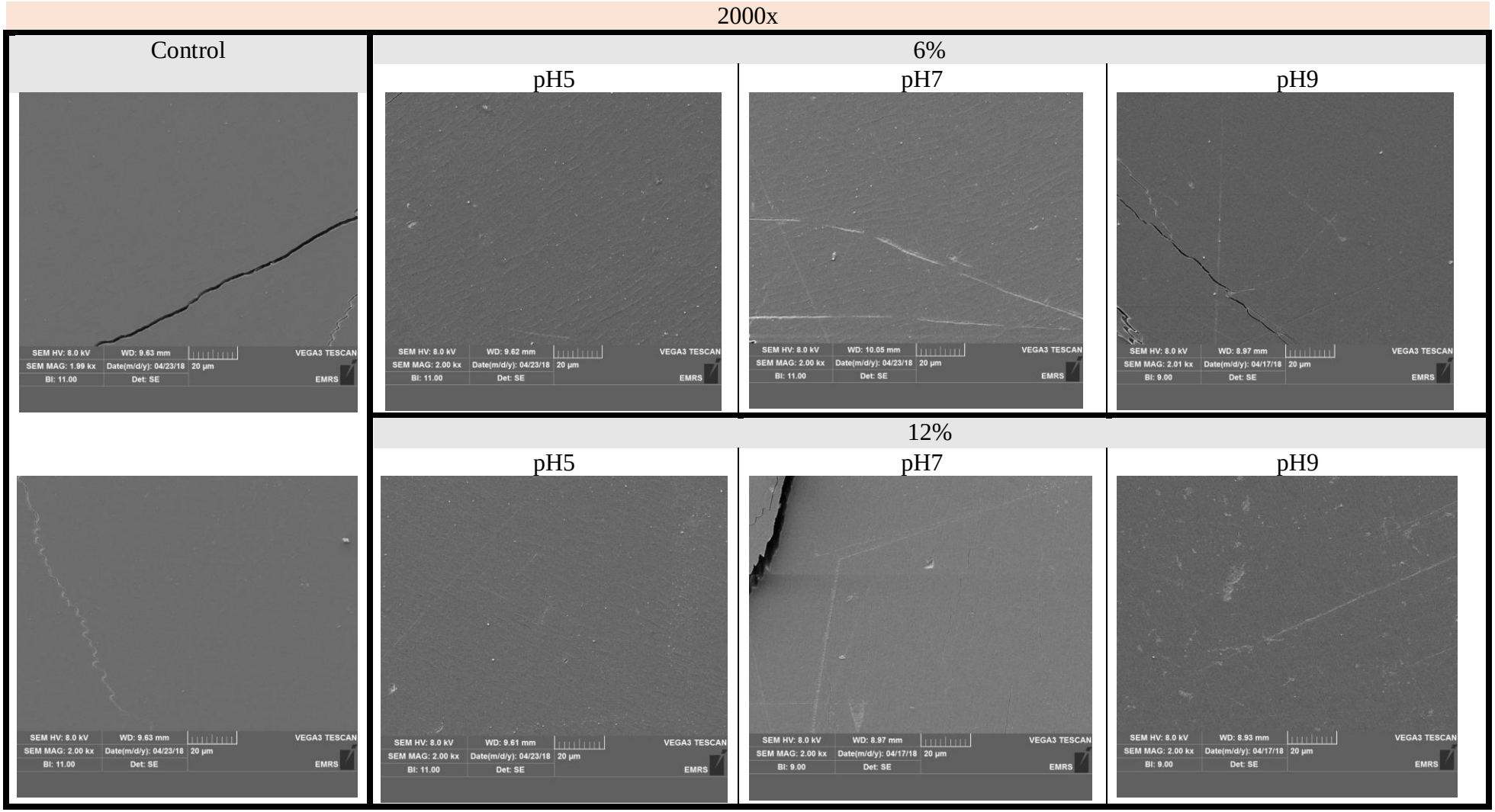


Figure 23 Scanning electron microscopy images of the control (left column), 6%HP (top row), and 12%HP (bottom row) groups under 2000x magnification. Whitened enamel appear more irregular in comparison to the control group. The exposure of enamel prismatic structure is evident in whitened enamel surfaces; visible as perikymata-like lines.

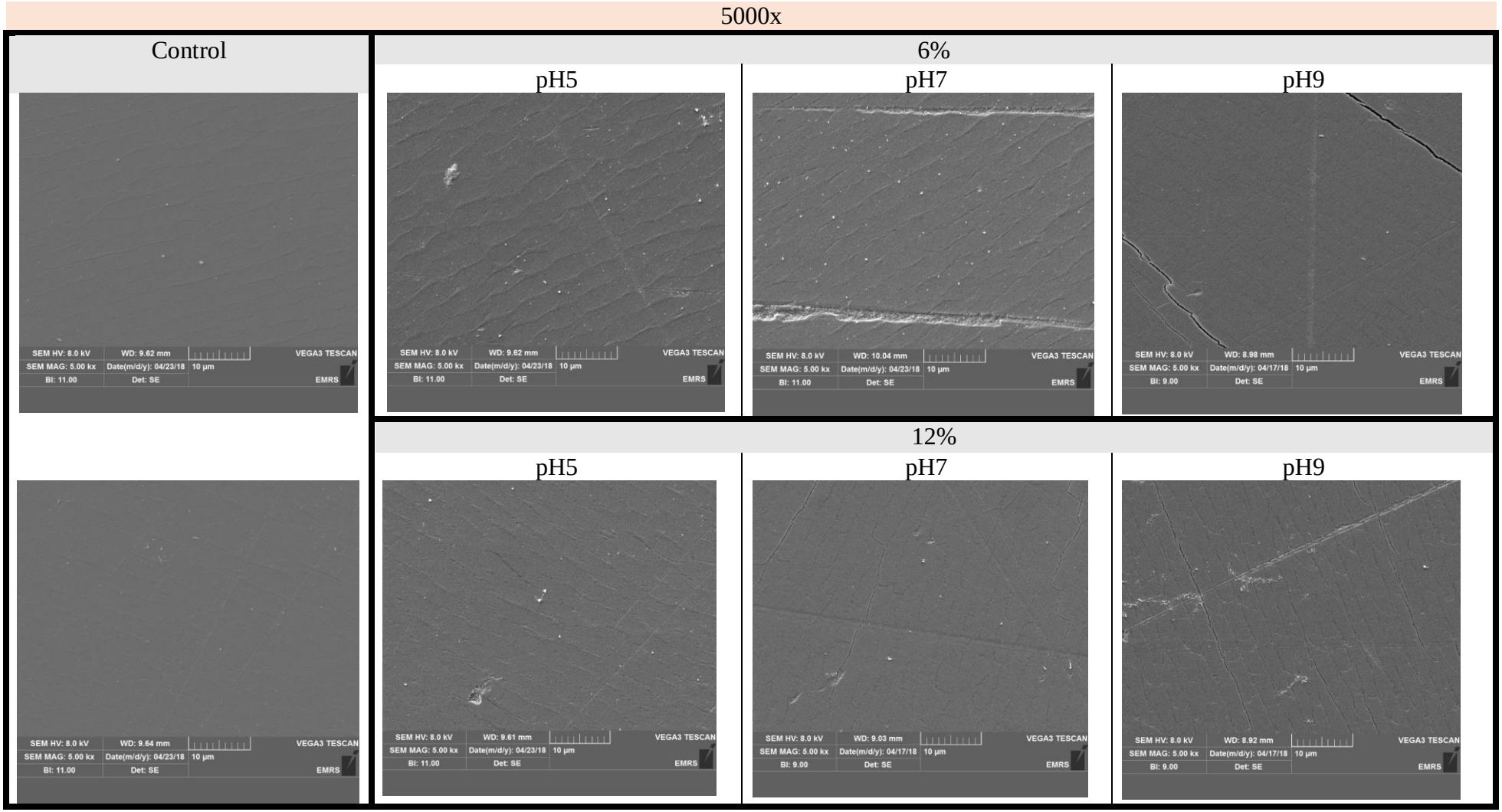


Figure 24 Scanning electron microscopy images of the control (left column), 6%HP (top row), and 12%HP (bottom row) groups under 5000x magnification. Whitened enamel appear more irregular in comparison to the control group. The exposure of enamel prisms was more pronounced under high magnification in whitened enamel samples as compared to the control group.

Discussion

The pilot study was conducted to investigate the effects of 6% and 12% HP with acidic, neutral, and alkaline pH values of 5, 7, and 9, on bovine enamel roughness, colour change, surface microstructure, and elemental composition. The 6% HP was selected for being the maximum allowed concentration to be used for dental whitening in the UK (GDC, 2011), while the 12% HP; being double the allowed concentration, would provide a meaningful and clinically relevant comparison. Enamel samples were treated for 2hrs/daily for 14 days which is in line with tray-based whitening protocols usually prescribed for 2-4 hours/daily, for 10-14 days (Stokes *et al.*, 1992; Kihn, 2007; Mondelli *et al.*, 2009).

The outcome of dental whitening procedures depends on the concentration and pH of the whitening agent, whitening duration, and the frequency the whitening agent is in contact with enamel chromogens (Dahl and Pallesen, 2003). Oxygen radicals released by HP react with organic chromogens present in enamel through an oxidation process which destabilises the chromogenic compound, and ultimately reduces tooth discolouration (Carey, 2014). Current results showed that colour change was directly proportional to the whitening duration and that more than 80% of the overall colour change (ΔE) in bovine enamel samples occurred after 7 days of whitening. According to a whitening study using 16% and 10% CP and 6.5% HP, the greatest enamel colour change was recorded after 7 days, followed by a minor increase in ΔE after 14 and 21 days of whitening (Delfino *et al.*, 2009). Current results have also shown that the higher HP concentration (12%) caused a greater whitening effect, which is in line with previously published research (Kawamoto and Tsujimoto, 2004; Fearon, 2007; Borges *et al.*, 2015). The diffusion rate of HP molecules is directly proportional to the concentration of the HP solution (Torres *et al.*, 2013; Cintra *et al.*, 2016). This means that higher concentrations of HP penetrate treated enamel surfaces more rapidly, which result in greater colour changes (Cintra *et al.*, 2016). On the other hand, HP solution pH did not significantly affect the degree of colour change in whitened enamel. HP solutions were reported to cause greater colour change at pH8 and pH7 (Xu *et al.*, 2011), pH9 (Torres *et al.*, 2014), and pH 4 and 7.5 (Sa *et al.*, 2012a). All three studies used HP with concentrations ranging from 17% to 38% which is higher than the concentrations used in this current study. Furthermore, according to Xu *et al.* and Torres *et al.* HP solutions were applied for two 30 minute cycles and two 10 minute cycles respectively. In regards to the study performed by Sa *et al.*, the acidic and neutral HP solutions were applied for 90 minutes. In addition to variations in HP concentrations between these studies, different whitening durations might have affected the resultant colour change. The decomposition rate of HP increases as the solution pH increases (Pędziwiatr, 2018). This means that the degradation rate of alkaline HP is greater than acidic and neutral HP solutions; requiring more

time to breakdown and release free oxygen radicals. Therefore, within a limited time frame a larger portion of the alkaline solution will degrade, and greater colour changes in treated enamel surfaces will occur as a result.

It has been reported that whitening treatments using HP lead to significant changes in enamel microstructure (McCracken and Haywood, 1996; Taube *et al.*, 2010; Coceska *et al.*, 2016). In addition, treating enamel with a whitening agent for 14 days was reported to cause surface alterations and the exposure of enamel prisms (Bitter, 1998). Indeed, qualitative analysis conducted in this pilot study revealed the exposure of enamel prisms in whitened bovine enamel, according to SEM images. Surface irregularities noted, were similar in enamel samples treated with 6% and 12%HP solutions. According to a study conducted by Abouassi *et al.*, there were no significant microstructural changes in enamel samples treated with 3.6% HP according to SEM images (Abouassi *et al.*, 2011). Images of enamel samples treated with 10% HP, on the other hand, revealed surface irregularities similar to results reported in this study.

In dental studies, Ra is the most frequently reported parameter to measure surface roughness (Abouassi *et al.*, 2011). Roughness average measurements help quantify surface changes in treated enamel samples. The results have shown that treatment with 6% and 12% HP with pH values of 5, 7, and 9, did not significantly change enamel Ra values in comparison to BL values and the control group. Roughness measurements were obtained using a stylus profilometer with a 5μm tip radius; this tip radius may have been too large to trace and measure intricate surface irregularities present on the nano-scale (Seitavuopio, 2006). Enamel treated with 7.5% HP with pH values of 5.1, for 14 days did not exhibit significant changes in Ra values according to stylus profilometer readings (Pinto *et al.*, 2004). On the other hand, Ra values significantly increased in enamel samples treated with 6%, 15%, and 35% HP with pH values of 3.5, 2.5, and 1.2, respectively, according to nano-measurements obtained using AFM (Rodrigues *et al.*, 2017). Differences could be explained by the highly acidic HP solutions used by Rodrigues *et al.*, which would in turn lead to greater mineral loss; causing irregular and rougher enamel surfaces (Derceli *et al.*, 2016). In addition, measuring enamel roughness on a nanoscale using a high resolution imaging system; an AFM for instance, could have provided more insight on enamel surface changes caused by HP by detecting minor surface irregularities too small to be detected by a stylus profilometer.

The effects of dental whitening on enamel chemical composition was examined by measuring atomic % of Ca, C, and P using EDX. Whitening enamel using acidic, neutral, and alkaline 6% and 12% HP resulted in no significant mineral loss. According to the literature, some studies have reported no significant mineral loss in whitened enamel samples (PARK *et al.*, 2004; Moreira *et al.*, 2017), while others recorded a significant reduction in enamel mineral content

after whitening (Efeoglu *et al.*, 2005; Al-Salehi *et al.*, 2007; Jiang *et al.*, 2008; Souza *et al.*, 2010). Differences could be attributed to different measuring techniques (Efeoglu *et al.*, 2005; Jiang *et al.*, 2008), excessive whitening durations (Al-Salehi *et al.*, 2007; Souza *et al.*, 2010), or the destructive preparation process of enamel samples for SEM/EDX; making comparisons between readings from the same enamel sample unachievable (Moreira *et al.*, 2017). Since there is a direct relationship between enamel mineral composition and microhardness (Pizani *et al.*, 2015; Moreira *et al.*, 2017), enamel microhardness would be a suitable supplementary measuring technique to reflect the elemental condition of treated enamel surfaces.

During the pilot study, a chemical reaction between remnants of HP in whitened enamel samples and the chloramine-T storage medium was evident. Mixing HP and chloramine-T was reported to cause a bimolecular reaction, resulting in the precipitation of chloramine-T salts (Coull *et al.*, 1935) (Figure 25). Therefore, changing the storage solution will be required to avoid unwanted chemical reactions and better represent oral environment conditions. Whitened enamel samples stored in artificial saliva were reported to produce similar physical and optical properties as samples stored in natural saliva (Zeczkowski *et al.*, 2015). Furthermore, in numerous published whitening studies, treated enamel samples were stored in artificial saliva at 37°C to simulate oral environment conditions (Basting *et al.*, 2005; Bayrak *et al.*, 2009; Grobler *et al.*, 2009; Majeed *et al.*, 2011b; Araujo *et al.*, 2013; Alaghemand *et al.*, 2015). Therefore, artificial saliva would be a suitable storage medium for treated enamel samples in future experiments.

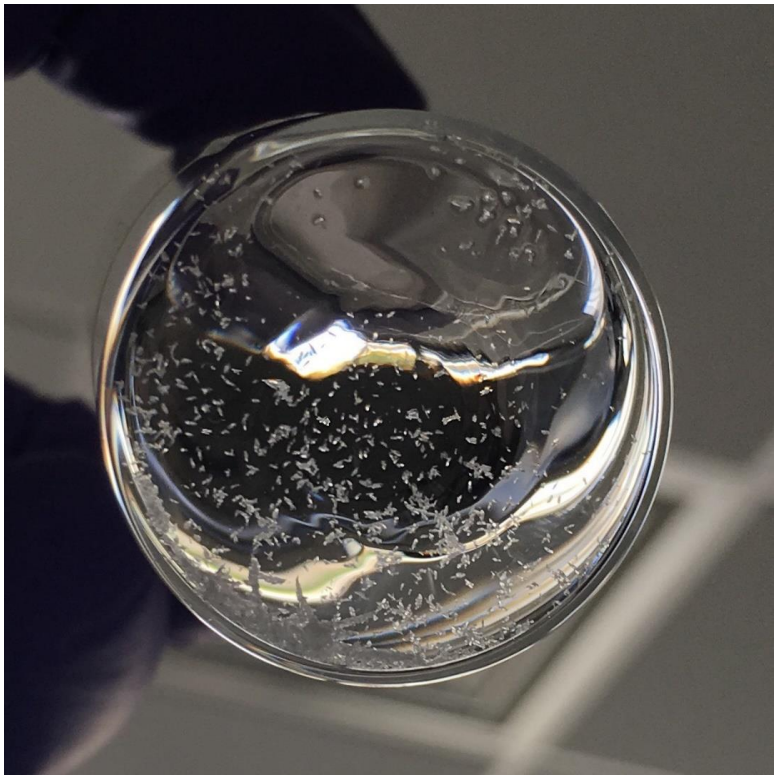


Figure 25 White precipitants observed at the bottom of all vials containing whitened enamel specimens stored in 1% Chloramine-T solutions.

Conclusion

Whitening treatments conducted in this pilot study resulted in significant changes in enamel colour; mostly recorded after the first 7 days of treatment. Whitened enamel samples showed surface irregularities according to SEM images in addition to the exposure of enamel prisms in comparison to the control group. In addition, whitening using 6% and 12%HP caused no significant changes in enamel Ra values and mineral composition. Therefore, substituting Ra micro-measurements with nano-measurements could provide a greater sensitivity to measure potential changes post whitening. In addition, supplementing elemental composition readings with microhardness measurements will be useful in reflecting enamel mineralisation after whitening treatments. Chloramine-T chemically reacted with HP remnants in whitened enamel samples, making it an unsuitable storage medium in whitening studies.

Recommendations

Based on results obtained from the pilot study the following recommendations were developed:

1- Measuring enamel roughness on the nano-scale.

2- Measuring the effect of whitening on enamel hardness.

3- Storing enamel samples in artificial saliva at 37°C.

4.2.6 Measuring enamel roughness on the nano-scale

Roughness measurements of enamel surfaces were obtained using an atomic force microscope (AFM) (NanoWizard® 3 NanoOptics AFM system, JPK Instruments, Berlin, Germany). Bovine enamel samples (n=4) were imaged under dry and wet conditions, in an effort to determine the most suitable measuring technique. Under dry conditions, a sharper more detailed image of enamel was recorded, illustrating the prismatic and interprismatic structures in comparison to the wet condition (Figure 26). Therefore, enamel Ra measurements were obtained under dry conditions in future experiments.

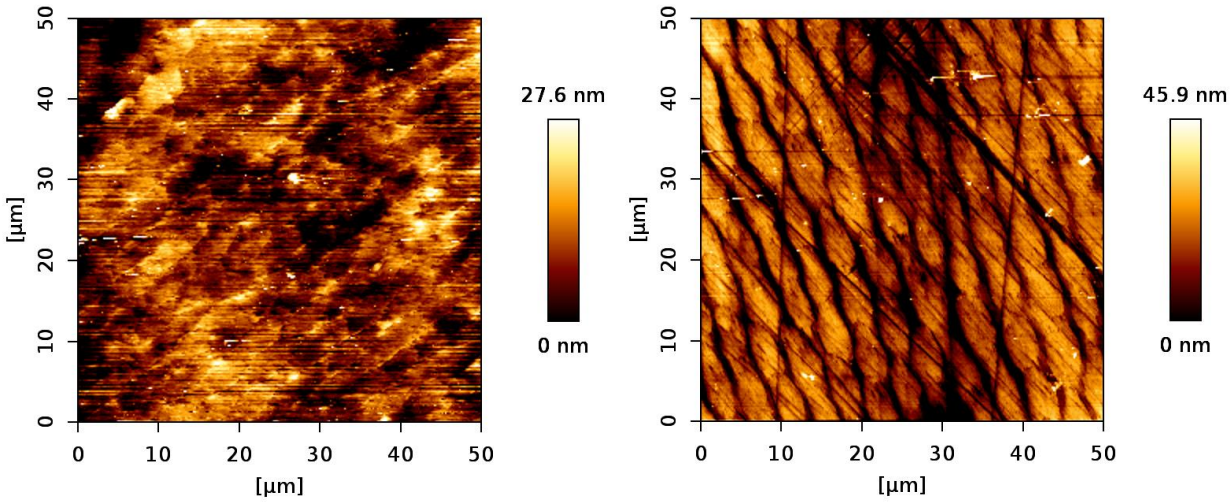


Figure 26 An AFM image of bovine enamel under a wet condition (left) and a dry condition (right). Note the superior quality of the image obtained under dry conditions.

4.2.7 Storage medium and storage temperature

Artificial saliva was made based on a previously published method (Earl *et al.*, 2011) using components listed in Table 10. Additionally, storage temperature was set at 37°C in accordance to oral environment conditions.

Artificial Saliva		
Components	Quantity	Manufacturer
Calcium chloride dihydrate	0.44g	Sigma-Aldrich©, Inc., Irvine, UK
Potassium chloride	2.24g	
Potassium dihydrogen phosphate	1.36g	
Sodium chloride	0.76g	
Mucin (porcine stomach mucin)	2.2g	
Potassium hydroxide	To adjust pH to 6.5	Fisher Scientific©, Leicestershire, UK
Distilled deionised water	1L	

Table 10 Constituents of the artificial saliva used in this study.

4.2.8 General Discussion

The aforementioned preliminary studies were performed to obtain HP solutions with different concentrations and pH values, test HP pH stability with time, select a suitable test substrate, and investigate the effects of whitening duration and HP concentration and pH on the selected test substrate.

The concentration of HP in commercially available whitening agents must not exceed 6%, according to the Cosmetic Products Enforcement Regulations in the UK (GDC, 2011; CPER, 2013). In addition, the American Dental Association (ADA) awards the seal of acceptance to products having the maximum concentration of 10% CP (3.5% HP) (ADA, 2008), and according to the national guidelines from WorkSafe in Australia, HP is considered a hazardous substance in concentrations above 5% (Walsh, 2000). However, according to previously published studies, the concentration of HP in commercially available products can reach up to 40% (Kwon *et al.*, 2013; Lubbadeh *et al.*, 2018), and at such high concentrations reactive oxygen radicals are able to cross the cell membrane and inflict damage to the treated tooth structure by causing genotoxicity and cytotoxicity (Tredwin *et al.*, 2006). Furthermore, the pH of commercially available products were reported to range from 2.39 to 11.13 (Price *et al.*, 2000; Freire *et al.*, 2009; Majeed *et al.*, 2011a; Jamshidian *et al.*, 2016). Therefore, to better reflect the wide range of HP based whitening products available in the market, concentrations obtained in this study ranged from 3% to 40%; adjusted to acidic, neutral, and alkaline pH values ranging from 3 to 10.

After achieving the desired HP concentrations and pH values as described in sections 4.2.1 and 4.2.2, test solutions were monitored using a pH meter to investigate their stability with time. According to the literature, the decomposition rate of HP increases as the solution pH increases (Pędziwiatr, 2018). It has been reported that at a pH of 11.5-12, the concentration of 800mg/L HP solution decreased by 12% after three hours of monitoring (Yazici and Deveci, 2010). This means, that at higher pH values the stability of the HP solution is reduced and rapid changes in pH and concentration occur as a result. This is in line with current results; showing rapid changes in pH values in the pH10 HP solution for up to three hours. Furthermore, significant changes in pH ($pH>\pm0.01$) mostly occurred after two hours of monitoring in all HP solutions. This helped determine the whitening duration in the study protocol as, to date, there are no published studies on the pH stability in HP solutions with concentrations ranging from 3% to 40%.

Bovine enamel is histochemically and anatomically similar to human enamel (Nakamichi *et al.*, 1983), with comparable calcium and carbonate levels and fluoride uptake, and similar responses to whitening (Yassen *et al.*, 2011). Therefore, polished bovine enamel samples were selected

as a test substrate in this study. Although no statistically significant differences were found between Ra values of polished and unpolished bovine enamel samples, the polished samples were consistently smoother according to SEM images obtained. Polishing procedures are commonly carried out in previously published research to minimise enamel surface variations and create standardised surfaces at BL (Alexandrino *et al.*, 2014; Pimenta-Dutra *et al.*, 2017; Sorozini *et al.*, 2018); by this providing more meaningful comparisons between treatment groups.

Based on results obtained from the pilot study, a number of modifications were made to the whitening protocol. The whitening duration was modified from 14 days to 10 days, as results have shown that more than 80% of colour changes occurred after 7 days of whitening, which is in accordance to the literature (Delfino *et al.*, 2009). This remains in line with tray based whitening protocols usually prescribed for 2-4 hours/daily, for 10-14 days (Stokes *et al.*, 1992; Kihn, 2007; Mondelli *et al.*, 2009). Furthermore, roughness measurements (Ra) were obtained using atomic force microscopy under dry conditions through a quantitative high-resolution imaging process reported to have great accuracy and precision down to the nano-scale (Eaton and West, 2010). It has been reported that dry imaging may improve the quality of images obtained from biological samples by eliminating the internal dynamic of living particles (Gaczynska and Osmulski, 2008). Indeed, upon comparison between bovine enamel samples imaged under dry and wet conditions, dry imaging provided a clearer image of enamel prismatic structure in comparison to wet imaging.

Enamel Vickers hardness values were additionally obtained. The direct relationship between enamel hardness and mineral composition make the assessment of enamel microhardness a suitable supplementary measuring technique to reflect the elemental condition of treated enamel samples (George *et al.*, 2015; Nematianaraki *et al.*, 2015; Pizani *et al.*, 2015; Moreira *et al.*, 2017; Mushashe *et al.*, 2018). In addition, similar to previously published whitening studies, treated enamel samples were stored in artificial saliva at 37°C to simulate oral environment conditions and avoid unwanted chemical reactions (Basting *et al.*, 2005; Bayrak *et al.*, 2009; Grobler *et al.*, 2009; Majeed *et al.*, 2011b; Araujo *et al.*, 2013; Alaghemand *et al.*, 2015).

4.3 Exposing whitened bovine enamel to dietary staining and erosion cycles

A number of experiments were undertaken to develop dietary staining and erosion cycles.

4.3.1 Dietary staining of whitened bovine enamel

Six bovine enamel samples were divided into two groups. The first group was whitened using 6% HP with a pH value of 5 for 2 hours, daily, for 10 days. The second group, the control, was treated with PBS (pH 7.4) following the same protocol. After treatment, enamel samples were immersed in coffee for three consecutive five-minute cycles, daily, for three consecutive days. Coffee was made by diluting 1.8g of a commercially available instant coffee (Nescafe© Original, Nestle, York, UK) in 200mL of boiling tap water (≥ 100°C). The beverage was then allowed to cool down to 60°C. The staining cycle was performed on a digital hot plate magnetic stirrer (VELP, Scientifica, Italy) at 60°C and the solution was agitated at 60 rpm. Colour measurements were obtained using a spectrophotometer (Ci62, X-Rite Europe GmbH, Regensdorf, Switzerland) at baseline and after each staining cycle, and samples were stored in artificial saliva at 37°C between treatments.

Results

Stain uptake was greater in whitened enamel samples as compared to the control group (Table 11) (Figure 27). Noticeable colour change occurred after the first staining cycle in day1 in the whitened enamel group and after the first staining cycle in day2 in the control group ($\Delta E > 2$). After three days of staining, colour change in whitened enamel samples reached up to 46%, while the control group showed a 33% colour change.

	Time (min.)	ΔE	
		Whitened	Control
Day1	5	2.4 (1.6)	1.4 (0.6)
	10	1.2 (0.8)	0.5 (0.3)
	15	1.8 (1.8)	1.1 (0.1)
Day2	5	3.0 (1.1)	2.5 (0.5)
	10	1.6 (0.6)	1.9 (0.7)
	15	1.9 (1.1)	0.7 (0.4)
Day3	5	5.6 (2.6)	1.4 (0.4)
	10	4.3 (2.1)	2.1 (0.9)
	15	3.7 (3.0)	1.9 (0.9)

Table 11 The overall colour change (ΔE) after three 5-minute staining cycles, repeated for three consecutive days. Values between parenthesis are standard deviations.

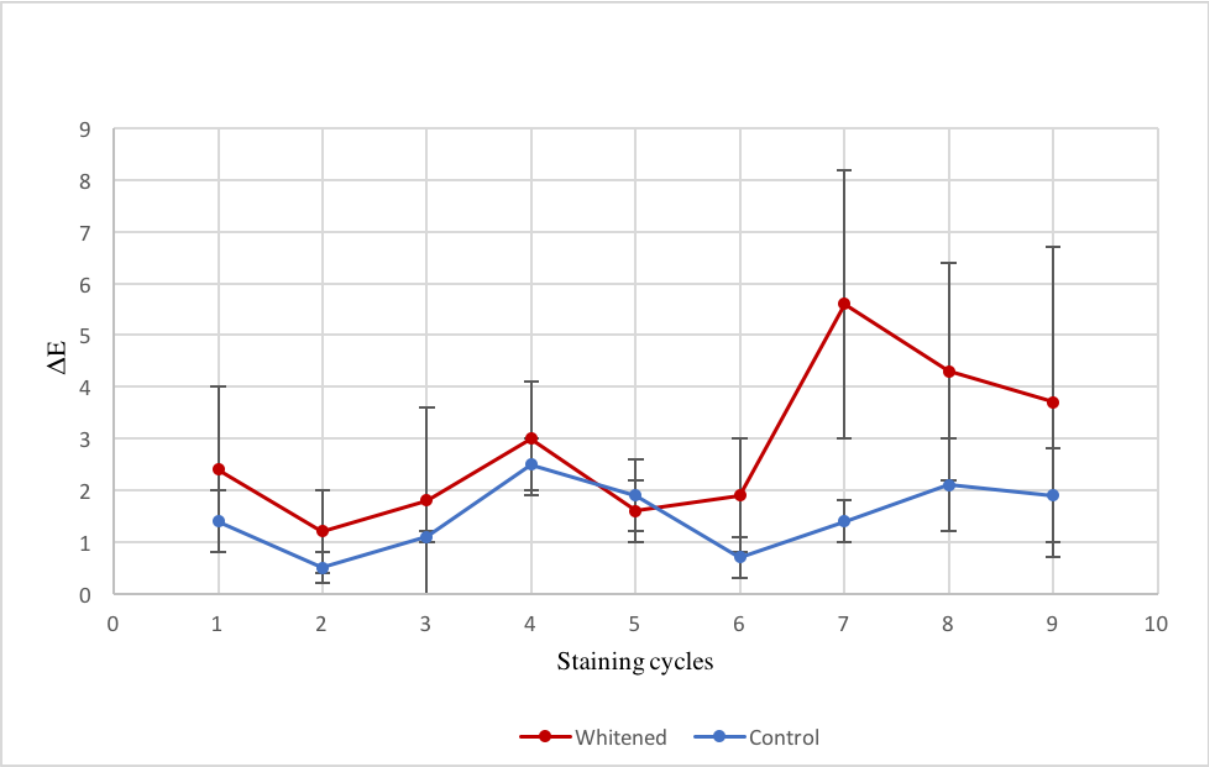


Figure 27 A representative graph of average ΔE values in whitened and control enamel after being subjected to a total of 9 staining cycles. Note the greater colour change in whitened enamel in comparison to the control. Error bars represent the variability of data.

4.3.2 Dietary erosion of whitened bovine enamel

Six bovine enamel samples were divided into two groups. The first group was whitened using 6% HP with a pH value of 5 for 2 hours, daily, for 10 days. The second group, the control, was treated with PBS (pH 7.4) following the same protocol. After treatment, enamel samples were immersed in a 0.3% citric acid solution for 15-minutes, daily, for three consecutive days. The citric acid solution was made by dissolving 0.3g of citric acid (Fisher Scientific©, Leicestershire, UK) in 100mL distilled water. The solution pH was then modified, using 0.5M NaOH, to form a pH value of 3.8. The erosion cycle was performed at room temperature and the solution was agitated at 60 rpm (VELP, Scientifica, Italy). Roughness and hardness measurements were obtained at baseline and after each erosion cycle using atomic force microscopy (NanoWizard® 3 NanoOptics AFM system, JPK Instruments, Berlin, Germany) and a using a universal test machine (Zwick Z 2.5, Zwick GmbH & Co., Ulm, Germany), respectively. Samples were stored in artificial saliva at 37°C between treatments.

Statistical analysis

Statistical analysis was performed using SigmaPlot for Windows version 13.0, Build 13.0.0.83 (Systat Software 2014®). Data not normally distributed ($P < 0.050$, Shapiro-Wilk) with no equality of variance ($P < 0.050$, Brown-Forsythe) were statistically compared using Kruskal-Wallis One Way Analysis of Variance on Ranks with an overall significance of 0.05. Normally distributed data were analysed using One Way Analysis of Variance with an overall significance of 0.05.

Results

Both groups showed a statistically significant increase in Ra, after erosion, in comparison to BL values (Table 12). Roughness average values were significantly greater in control enamel samples in comparison to the whitened enamel group after the first and third erosion cycles (Figure 28). In regards to enamel hardness, a statically significant decrease in mean Vickers hardness values (HV) were recorded in whitened enamel samples after the third erosion cycle in comparison to baseline values (Table 13). No statistically significant changes in HV values were recorded in the control group after all erosion cycles. Hardness values in whitened enamel samples were significantly lower than the control group after the third erosion cycle (Figure 29).

Erosion cycles	Ra (IQR)	
	Whitened	Control
Baseline	6.8 (3.9)^{a,A}	5.4 (2.7)^{a,A}
Cycle1	35.7 (124.9)^{a,B}	149.9 (152.6)^{b,B}
Cycle2	124.4 (223.9)^{a,B}	171.8 (39.8)^{a,B}
Cycle3	142.3 (87.0)^{a,B}	177.5 (59.8)^{b,B}

Table 12 Median Ra values at BL and after three 15 minute erosion cycles. Values between parenthesis are Inter-quartile ranges (IQR). Different lowercase superscript letters indicate statistically significant differences between columns. Different uppercase superscript letters indicate statistically significant differences between rows.

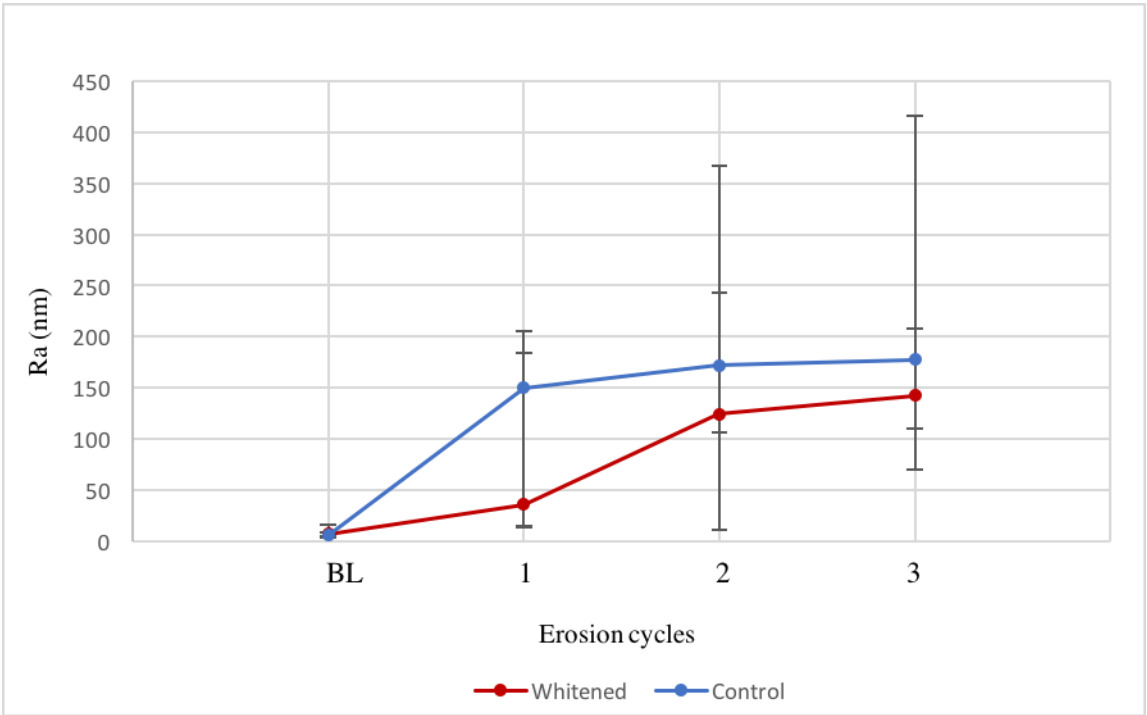


Figure 28 A representative graph of Median Ra values at BL and after three 15 minute erosion cycles. Note the dramatic increase in Ra in the control group in comparison to the whitened enamel group. Error bars represent the variability of data.

Erosion cycles	HV (SD)	
	Whitened	Control
Baseline	269.3 (44.0)[a,A]	301.2 (54.5)[a,A]
Cycle1	221.9 (83.6)[a,A]	268.0 (73.5)[a,A]
Cycle2	197.4 (60.4)[a,A]	264.1 (117.7)[a,A]
Cycle3	164.7 (14.3)[a,B]	211.1 (35.6)[b,A]

Table 13 Mean Vickers hardness values at BL and after three 15 minute erosion cycles. Values between parenthesis are standard deviations. Different lowercase superscript letters indicate statistically significant differences between columns. Different uppercase superscript letters indicate statistically significant differences between rows.

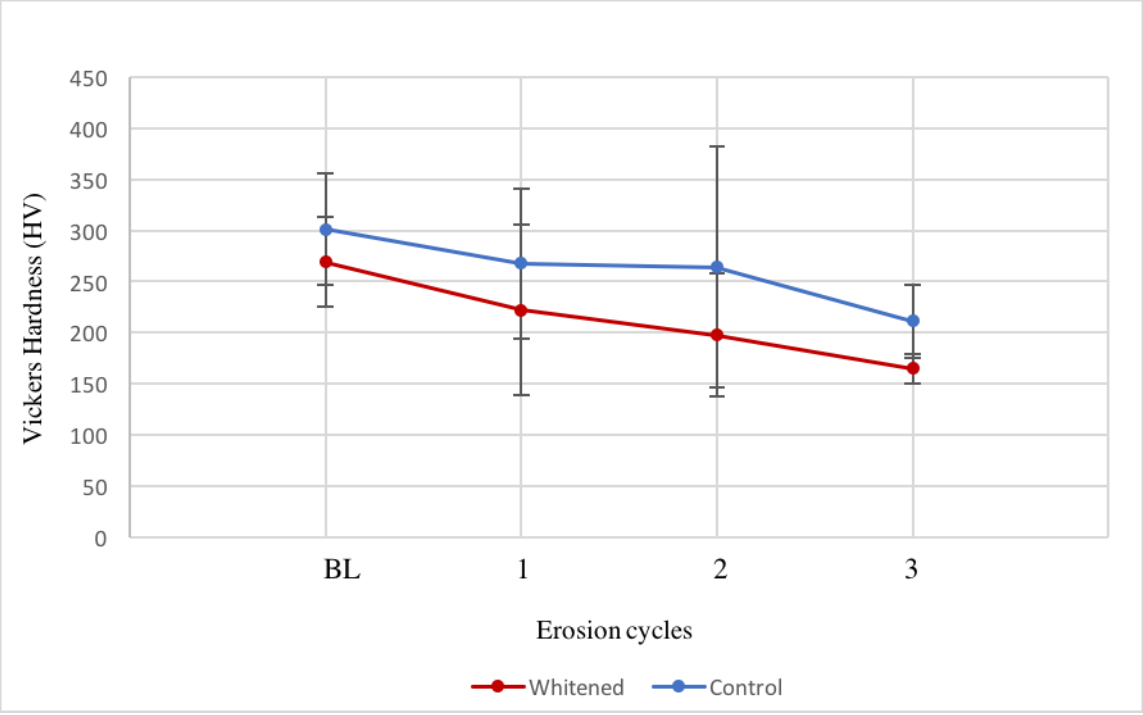


Figure 29 A representative graph of Mean HV values at BL and after three 15 minute erosion cycles. Note the gradual reduction in hardness after each erosion cycle. Error bars represent the variability of data.

4.3.3 General Discussion

Erosion and staining cycles were designed to investigate the effects of dietary acids and stains on whitened bovine enamel. The three-day cycling protocol helped assess the impact of single and repeated exposures by simulating cumulative dietary intake using what is usually consumed on a daily basis (West *et al.*, 2000; Mullan, 2018). Treated enamel samples were eroded using 0.3% citric acid with a pH value of 3.8 which is the most commonly found dietary acid in natural and commercial products, such as orange juice (Hughes *et al.*, 2000; Austin *et al.*, 2010; Shellis *et al.*, 2011). During the erosion cycle the solution was agitated at 60 rpm to simulate erosive challenges in the oral environment which are unlikely to be static (Mullan, 2018). The duration of each erosion cycle was set to 15 minutes as it has been reported in the literature that early erosive changes in terms of enamel roughness and hardness occur after 15 minutes of exposure (Meurman and Frank, 1991; Mullan, 2018).

Eroded enamel samples showed a significant increase in Ra, which is in line with previously published studies (Barac *et al.*, 2015; Mullan, 2018). The increase in roughness is caused by enamel mineral loss; resulting in irregular and deformed enamel surfaces (Derceli *et al.*, 2016). After three erosion cycles, changes in Ra were greater in the control group in comparison to the whitened enamel group. This could be attributed to the softening of the enamel surface after being exposed to repeated erosive challenges; whitening and dietary erosion, resulting in lower roughness readings (Lopes *et al.*, 2002). According to Borges *et al.*, following exposure to a soft drink with a pH value of 2.8, erosive changes were greater in sound bovine enamel samples (3.37μm) in comparison to enamel samples whitened using 35%HP (2.89μm) (Borges *et al.*, 2012a). Exposing enamel to HP could provoke surface alterations; allowing the erosive solution to deeply penetrate into the treated enamel surface and increase its susceptibility to erosive changes in the form of enamel softening.

Vickers hardness readings confirm the aforementioned conclusions. After the third erosion cycle, a significant reduction in Vickers hardness values in whitened bovine enamel was observed. This was not the case, however, in the eroded control group, showing no significant changes in hardness values after all erosion cycles. In other words, subjecting whitened enamel samples to three 15-minute erosion cycles caused statistically significant reductions in enamel hardness values. According to Zanet *el al.*, whitened enamel is more susceptible to erosion in comparison to unwhitened enamel (Zanet *et al.*, 2011). They reported a greater hardness reduction in whitened enamel samples in comparison to the control group after being subjected to a 5-minute erosion cycle, using beverages containing citric, phosphoric, tartaric, maleic, and tannic acids, daily, for 7, 14, and 21 days. Greater reductions in microhardness values were additionally reported in enamel samples whitened using 35% HP then exposed to an erosive

beverage for 5 minutes, daily, for 7 and 14 days in comparison to a control group (Vivek *et al.*, 2014). Whitening procedures followed by exposure to dietary erosion lead to greater enamel mineral loss; resulting in rapid reductions in hardness readings.

Since coffee is a popular beverage, it was selected for the cyclic dietary staining (Mussatto *et al.*, 2011). The beverage was prepared in accordance to manufacturer's instructions and allowed to cool to 60°C which is the mean preferred temperature for hot beverages (Lee and O'Mahony, 2002; Brown and Diller, 2008). Again, the solution was agitated at 60 rpm to resemble oral environment conditions (Mullan, 2018), and each staining cycle was set to 15 minutes per day which is the mean consumption time for a cup of coffee (Ertas *et al.*, 2006).

Results revealed greater stain uptake in whitened enamel as opposed to the control group which is expected as HP increases enamel roughness, and as a consequence its susceptibility to dietary staining (Pinto *et al.*, 2004; Yeh *et al.*, 2005; Bistey *et al.*, 2007). This was confirmed in a study conducted by Azer *et al.*, reporting a greater dietary stain uptake in enamel samples whitened using 20% CP in comparison to the control unwhitened enamel group (Azer *et al.*, 2011). This was explained by the increase in whitened enamel roughness, and consequent subsurface penetration of dietary pigments. Furthermore, whitened enamel samples were reported to have greater stain uptake following exposure to cigarette smoke in comparison to unwhitened enamel (Públio *et al.*, 2013). According to this study, micro-porosities, irregularities, and depressions were present in enamel treated with 35%HP, which contributed to the significant stain uptake.

4.4 Conclusion

Tests described in this chapter were essential in planning and structuring the research protocol. Different HP concentrations and pH values were obtained and pH changes were monitored with time. The selected acidic, neutral, and alkaline pH values of 5, 7, and 9 were stable for up to 2 hours. In addition, results have shown that more than 80% of changes in enamel ΔE occurred after 7 days of whitening. Therefore, modifications were made to the whitening protocol by changing the whitening duration to 10 days, incorporating a quantitative high-resolution imaging process for Ra measurements, and recording enamel Vickers hardness to reflect the elemental conditions of treated surfaces.

Dietary staining and erosion cycles were designed to assess their impact on whitened enamel. Whitened enamel samples were more susceptible to dietary staining; exhibiting greater stain uptake, in comparison to the control group. However, the eroded control group had rougher surfaces than the eroded whitened enamel. This was attributed to the softening of whitened/ eroded enamel samples which was reflected by the significant reduction in their recorded HV readings.

Chapter 5. Phase I: The effects of hydrogen peroxide concentration and pH on enamel

5.1 Introduction

Dental whitening is an effective, minimally invasive, and safe treatment for improving patients smile aesthetics. Therefore, to get the greatest colour change with minimum side effects, one must consider key influencing factors such as the concentration and pH of the whitening product (Soares *et al.*, 2016a). To date, published *in-vitro* investigations concerning the impact of HP concentration and/or pH are limited and results from these studies are difficult to compare as the majority use commercially available products which contain various additives; possibly affecting the accuracy and reliability of any conclusions drawn (Sa *et al.*, 2012a; Trentino *et al.*, 2015; Cvikl *et al.*, 2016). Moreover, different whitening durations may have contributed to the lack of consistency in results acquired from these studies (Sun *et al.*, 2011; Xu *et al.*, 2011) and for that reason, controversy still exists around estimating the magnitude of impact HP concentration and pH have on whitened enamel.

In the present study the combined effects of HP concentration and pH have been evaluated and assessed. Concentrations tested started from 6%; being the maximum allowed HP concentration to be used for dental whitening in the UK (GDC, 2011), up to 40% HP which is a concentration present in commercially available whitening products (Acuña *et al.*, 2019) and excessively applying such high concentrations of HP might irreversibly damage treated enamel as a consequence (Lewinstein *et al.*, 2004; Jiang *et al.*, 2008). An intermediate concentration value of 20% HP has been additionally selected to assess the effects of gradual concentration elevation on bovine enamel, qualitatively and quantitatively. Each HP concentration was adjusted to form three pH values: an acidic (pH5), a neutral (pH7), and an alkaline (pH9).

5.2 Study aims

1- Study the effects of acidic, neutral, and alkaline HP in relation to bovine enamel colour, roughness, hardness, mineral composition, and surface quality.
2- Study the effects of low, intermediate, and high HP concentrations in relation to bovine enamel colour, roughness, hardness, mineral composition, and surface quality.

5.3 Materials and methods

Bovine enamel samples (Intertek© group plc., Cheshire, UK) (n=50) were whitened using three HP concentrations of 6wt.%, 20wt.%, and 40wt.% (Sigma-Aldrich, Inc., Irvine, UK), (n=15 per concentration) with pH of 5, 7, and 9 (n=5 per pH), for 2 hours, daily for ten days. HP concentrations and pH values were obtained as previously described in sections 4.2.1 and 4.2.2. A control group (n=5) was treated with Dulbecco's phosphate buffered saline (PBS) (Gibco©, Paisley, UK) (pH 7.4).

Bovine enamel samples were stored in 1% chloramine-T (Sigma-Aldrich, Inc., Irvine, UK) for one week before the start of the experiment. After which all samples were stored in artificial saliva (AS) at 37°C (Hot box oven with fan, Gallenkamp©, Loughborough, UK) and refreshed every other day. Before and after each treatment, all bovine enamel samples were rinsed for 10 seconds with PBS then thoroughly dried to minimise cross contamination. All samples were stored individually throughout the study in clear type 3 soda lime glass vials (Fisherbrand™, Fisher Scientific©, Leicestershire, UK).

Measurements of enamel roughness, hardness, and colour were investigated before and after treatment. Qualitative evaluation of enamel microstructure and quantitative assessment of mineral composition were obtained after treatment.

5.3.1 Enamel surface topography and roughness (Ra)

The characterisation of enamel surface topography was performed using atomic force microscopy (AFM) (NanoWizard® 3 NanoOptics AFM system, JPK Instruments, Berlin, Germany) (Figure 30). High-resolution 3-D images of enamel were captured through tracing and mapping along the x, y, and z axis and processed using JPK data processing programme (Version 6.1.116, JPK Instruments). Three AFM images were obtained per sample, under dry conditions, using an etched silicon probe (RTESP-525, Bruker©, Camarillo, CA) with a resonant frequency of 525 kHz. The AFM was set to QI™ mode and measurements were obtained over a 50×50µm^2 area at 1500nN contact load, 1000nm Z length, and a resolution of 256×256 pixels. Three enamel samples were imaged from each pH group and enamel Ra was calculated by averaging Ra readings obtained from 5 parallel lines per image along the fast scan axis. Surface Ra denotes the *arithmetic* mean of the roughness profile and defined as the deviation of profile heights (peaks and valleys) in relation to the mean line across the profile; calculated using the following equation:

$$\mathcal{R}a = \frac{1}{n}\sum_{i=1}^{n} |y_i|$$

Where n is the number of points detected along the traced surface and y_i is the vertical distance between a data point i and the mean line (DeGarmo *et al.*, 1997).

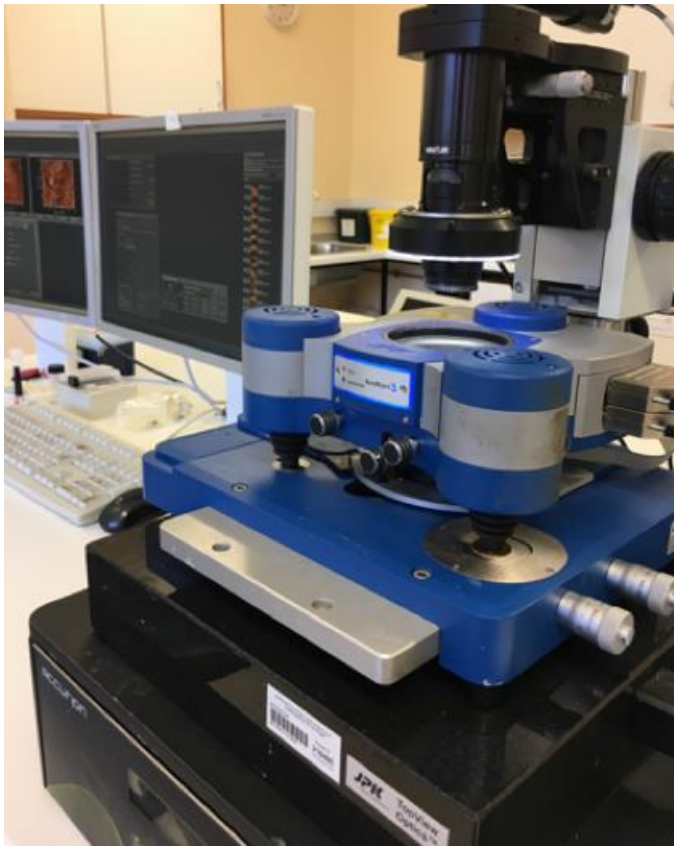


Figure 30 NanoWizard® 3 NanoOptics AFM system (JPK Instruments, Berlin, Germany).

5.3.2 Measuring enamel Vickers hardness (HV)

Vickers hardness measurements were obtained from all enamel samples using a universal test machine (Zwick Z 2.5, Zwick GmbH© & Co., Ulm, Germany) (Figure 31). Three measurements were recorded per sample using a diamond indenter with an approaching speed of 0.05mm/min and an applied load of 200g. The load is maintained for 20 seconds before the indenter is withdrawn at a speed of 0.1mm/min. The ratio between the applied load and the area indented is then used to calculate the sample's Vickers hardness using the following equation:

$$VH = \frac{1.854P}{d^2}$$

Where 1.854 is calculated from the geometry of the indenter, P is the applied indentation load in Newton and d is the diagonal length of indentation measured in mm (Chuenarrom *et al.*, 2009).

Figure 31 Universal micro-hardness test machine (Zwick Z 2.5, Zwick GmbH & Co., Ulm, Germany)

5.3.3 Measuring enamel colour

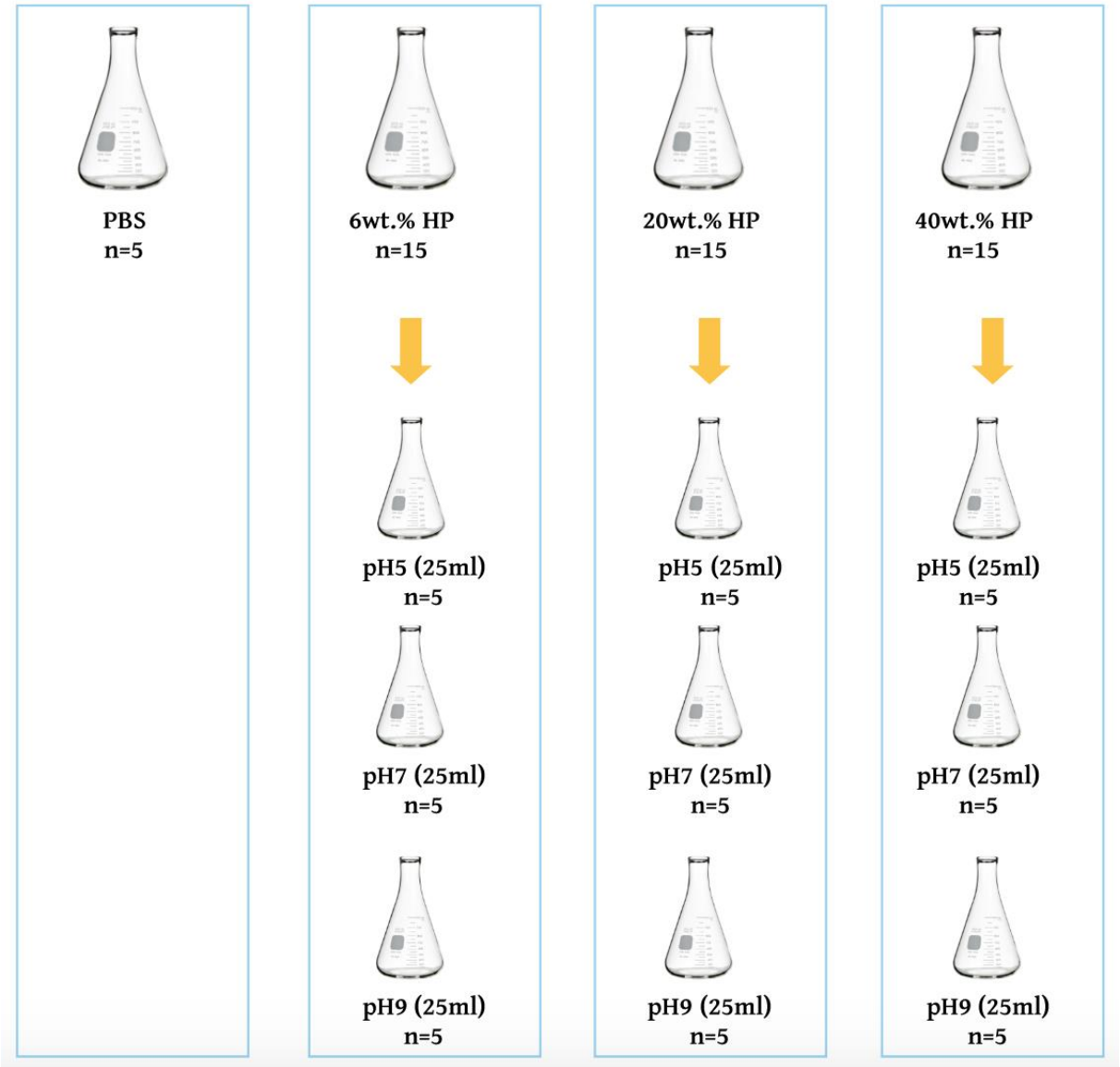
Colour measurements were obtained from all enamel samples using a spectrophotometer with a 4mm aperture (Ci62, X-Rite Europe GmbH, Regensdorf, Switzerland). Following the calibration of the device according to manufacturer's instructions, colour measurements were obtained using the CIE Lab system with a CIE standard illumination setting (D65/10°) corresponding to midday average light, viewed at a standard 10° angle. Three independent readings were taken for each sample against a white background and ΔE was calculated as described in section 4.2.5.

5.3.4 Measuring mineral composition and surface microstructure

Quantitative evaluation of mineral content in treated enamel samples (n=2 per pH) was undertaken using energy dispersive x-ray spectroscopy (EDX) set at 10 kV accelerating voltage (Bruker Xflash 6130, Bruker®, Camarillo, CA) and qualitative assessment of enamel microstructure was performed using Tescan Vega 3LMU SEM (Tescan Vega SEM LMU, Tescan, Cambridge). Samples were prepared for EDX and SEM as described in Section 4.2.4. Images were firstly obtained using SEM, and processed using its relevant TESCAN software under low, medium, and high magnifications ranging from 30x to 5000x. After which surface mapping was performed using EDX and mineral content was generated using Quantax Esprit 2.1 software (Bruker®, Camarillo, CA).

5.4 Study Design

The programme of work was designed as shown below:



5.5 Statistical Analysis

Statistical analysis was performed using Sigma Plot© for Windows version 13.0 build 13.0.0.83 (Systat Software 2014®). Statistical analysis of data was performed using Kruskal-Wallis One Way Analysis of Variance on Ranks for non-parametric data, while normally distributed data were analysed using One Way Analysis of Variance. Normality tests were performed using Shapiro-Wilk, and equal variance was tested using Brown-Forsythe. Pairwise multiple comparisons were done using Tukey test with an overall significance of 0.05.

5.6 Results

5.6.1 Whitening using 6% Hydrogen Peroxide

Enamel surface topography

Enamel exposed to HP showed an overall smooth and un-affected surfaces similar to the control group, with minor debris shown as white spikes. Differences are difficult to see due to the differences in z-scale in the images (Figure 32).

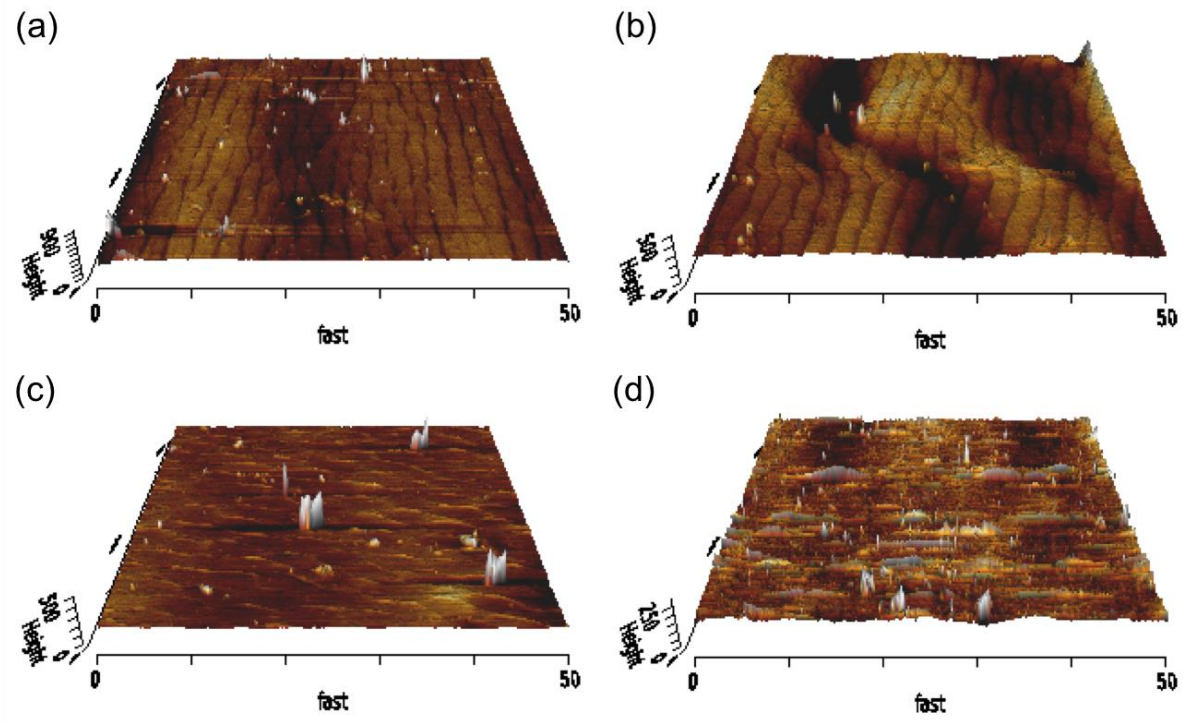


Figure 32 Representative 3D images of a control bovine enamel sample (a), treated with pH5 6% HP (b), treated with pH7 6% HP (c), and treated with pH9 6% HP (d). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed.

Enamel roughness (Ra)

After treatment with pH9 HP, enamel Ra increased by 49% (P≤0.05) (Figure 33) (Table 14). Treatment with pH5 and pH7 HP caused no significant changes in enamel Ra (P>0.05). Roughness values obtained in enamel treated with pH5 HP were significantly lower than those obtained in enamel samples treated with pH7 HP and pH9 HP solutions (P≤0.05).

	Ra (nm)	
	Before	After
pH5	6.1 (5.6)^{a,A}	5.1 (3.8)^{a,A}
pH7	7.8 (2.4)^{a,A}	8.6 (5.3)^{a,B}
pH9	6.8 (3.0)^{a,A}	10.1 (4.8)^{b,B}
Control	7.6 (2.8)^{a,A}	6.1 (2.0)^{a,A,B}

Table 14 Median and inter-quartile (IQR) values of enamel Ra before and after treatment using pH5, pH7, and pH9 6%HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

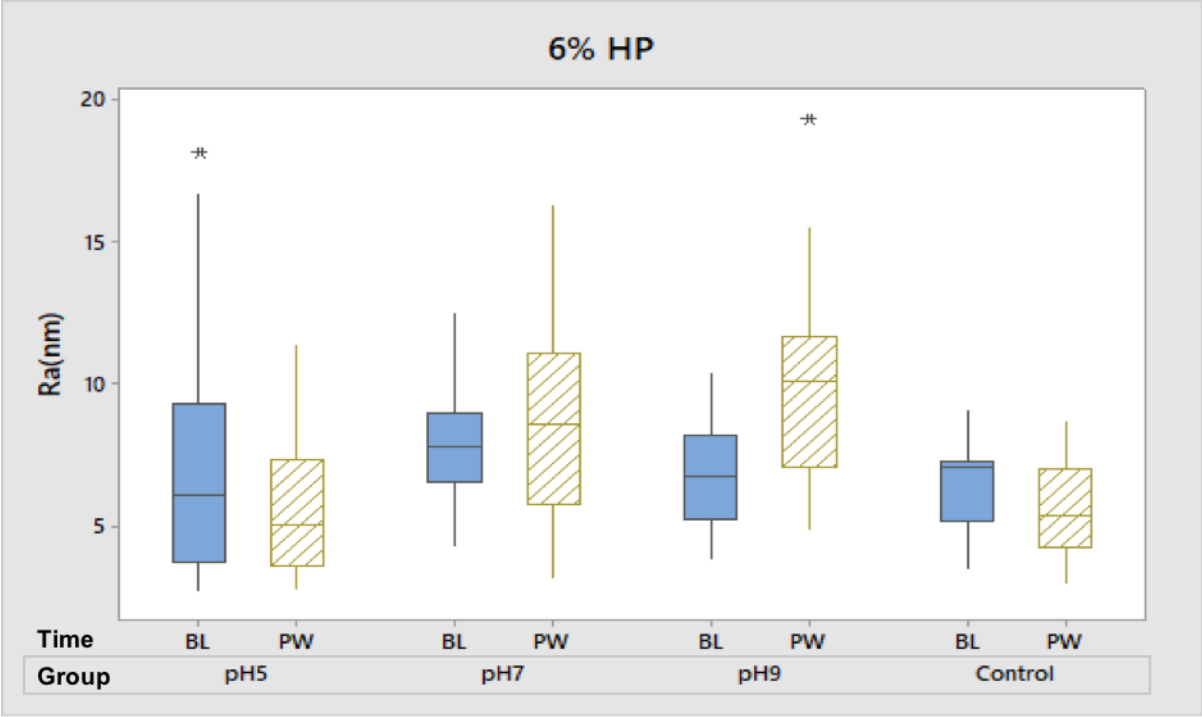


Figure 33 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 6%HP in addition to a control group.

Enamel hardness (HV)

Treatment with pH5, pH7, and pH9 HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 15) (Figure 34). Greatest reduction in hardness occurred in enamel treated with pH9 HP by 25.7%, followed by pH7 HP by 23%, and pH5 HP by 19%. After whitening treatments, enamel hardness did not significantly differ between treatment groups (P>0.05).

	HV (H_{IT})	
	Before	After
pH5	336.0 (21.4)^{a,A}	271.7 (83.5)^{b,A}
pH7	329.3 (42.6)^{a,A}	253.4 (78.9)^{b,A}
pH9	333.7 (28.0)^{a,A}	247.9 (46.3)^{b,A}
Control	323.1 (97.4)^{a,A}	256.1 (95.4)^{a,A}

Table 15 Median (IQR) values of enamel Vickers hardness before and after treatment using pH5, pH7, and pH9 6%HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

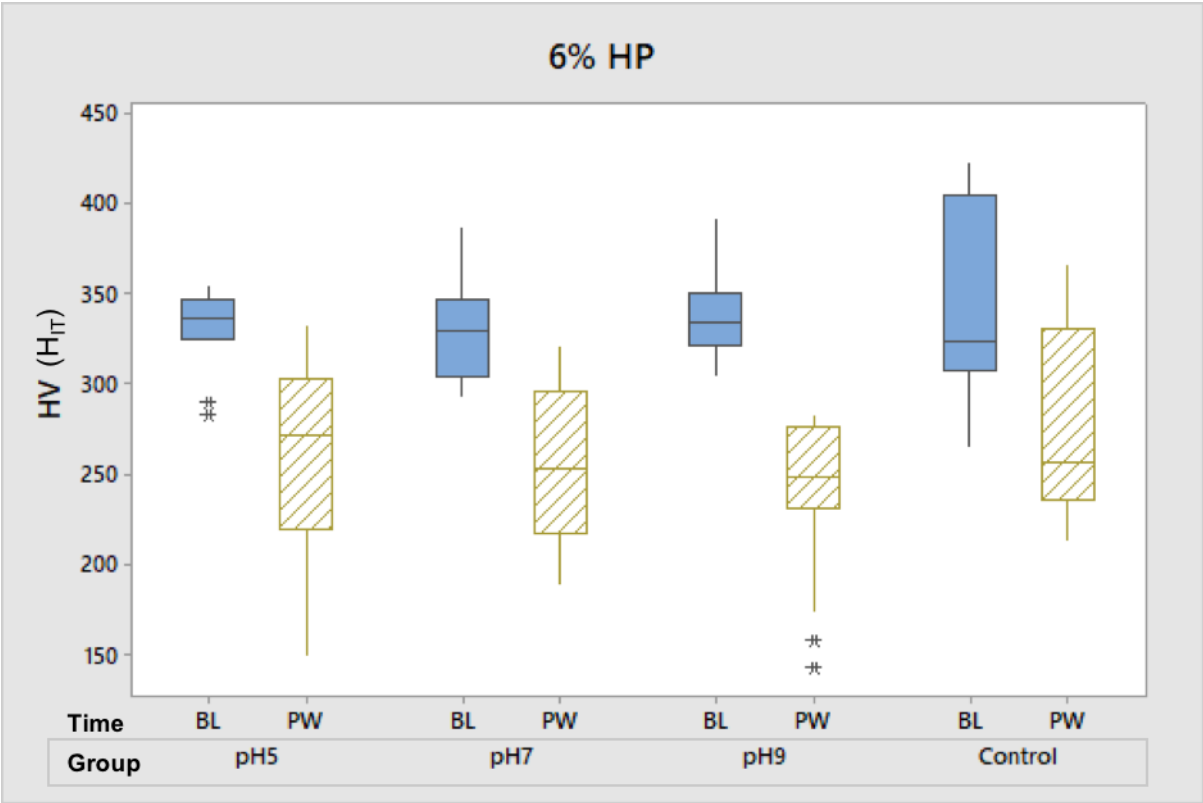


Figure 34 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 6%HP in addition to a control group.

Colour change

The greatest change in colour (ΔE) occurred in enamel treated with pH5 HP (Table 16). Enamel treated with pH7 and pH9 HP showed similar ΔE values, while the control group did not show any significant changes in colour (ΔE≤2). Treatment with pH5 HP caused a significant increase in enamel lightness (L*), a significant decrease in (a*) values away from the red and towards the green spectrum, and a significant decrease in (b*) values away from the yellow towards the blue spectrum (P≤0.05). Treatment using pH7 and pH9 HP caused no significant change in enamel lightness (P>0.05). Both, on the other hand, caused a significant decrease in the (a*) value towards the green spectrum and a significant decrease in (b*) values towards the blue spectrum (P≤0.05). Control enamel samples showed no significant changes in all colour parameters (L*), (a*), and (b*) (P>0.05). Changes in L*, a*, and b* values were not significantly different between whitened enamel samples (P>0.05).

ΔE			
pH5	pH7	pH9	Control
9.5 (1.5)^{a}	8.4 (1.7)^{a}	8.4 (1.2)^{a}	1.6 (1.1)^{b}
L* value			
	Before	After	
pH5	83.0 (1.7)^{a,A}	85.9 (1.0)^{b,A}	
pH7	83.3 (1.4)^{a,A}	84.9 (1.5)^{a,A}	
pH9	82.7 (2.2)^{a,A}	85.3 (1.9)^{a,A}	
Control	80.8 (1.3)^{a,A}	82.2 (0.8)^{a,B}	
a* value			
	Before	After	
pH5	-0.8 (0.3)^{a,A}	-1.4 (0.2)^{b,A}	
pH7	-0.7 (0.2)^{a,A}	-1.4 (0.1)^{b,A}	
pH9	-0.9 (0.3)^{a,A}	-1.4 (0.1)^{b,A}	
Control	-0.8 (0.4)^{a,A}	-0.6 (0.5)^{a,B}	
b* value			
	Before	After	
pH5	8.9 (1.5)^{a,A}	-0.1 (0.2)^{b,A}	
pH7	8.6 (1.1)^{a,A}	0.5 (0.5)^{b,A}	
pH9	8.3 (1.6)^{a,A}	0.5 (0.6)^{b,A}	
Control	9.8 (1.0)^{a,A}	11.7 (1.8)^{a,B}	

Table 16 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH5, pH7, and pH9 6% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

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Mineral composition

After treatment with pH5, pH7, and pH9 HP, atomic % of enamel calcium, phosphorus, and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 17).

Group	Element	Atomic %
pH5	Ca	27.0 (0.8)
	P	19.9 (0.1)
	C	13.8(0.4)
pH7	Ca	28.5 (0.3)
	P	20.4 (0.2)
	C	12.7(0.1)
pH9	Ca	27.7 (1.2)
	P	20.1 (1.0)
	C	13.0 (0.1)
Control	Ca	25.6 (1.2)
	P	20.4 (0.9)
	C	13.4 (0.5)

Table 17 Mean atomic % values and standard deviation (SD) of Calcium, Phosphorous, and Carbon in enamel treated using pH5, 7, and 9 of 6% HP in comparison to a control group.

Surface morphology

Control enamel samples and samples treated with pH5 and pH7 HP had an overall smooth surface with no visible enamel damage. Conversely, enamel treated with pH9 HP showed minor surface deformation and visible surface debris. Enamel cracks were visible in all imaged surfaces (Figure 35-38).

pH5

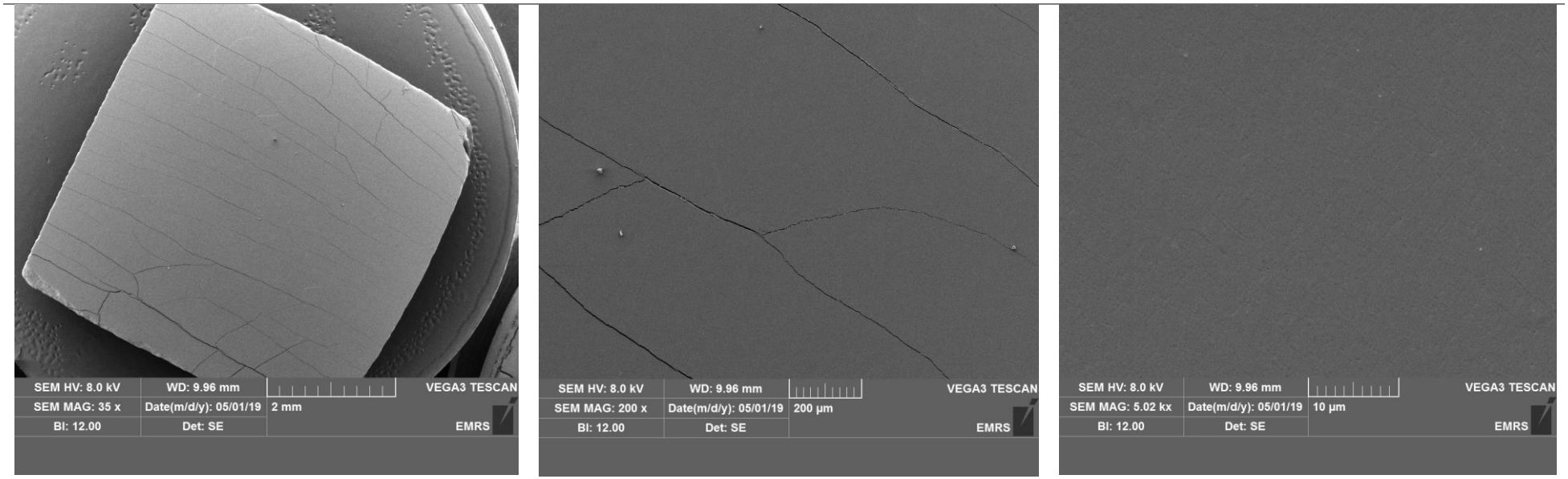


Figure 35 Three SEM images under low, medium, and high magnification of enamel treated with pH5 6% HP. Note the overall smooth surface with visible cracks under low magnification.

pH7

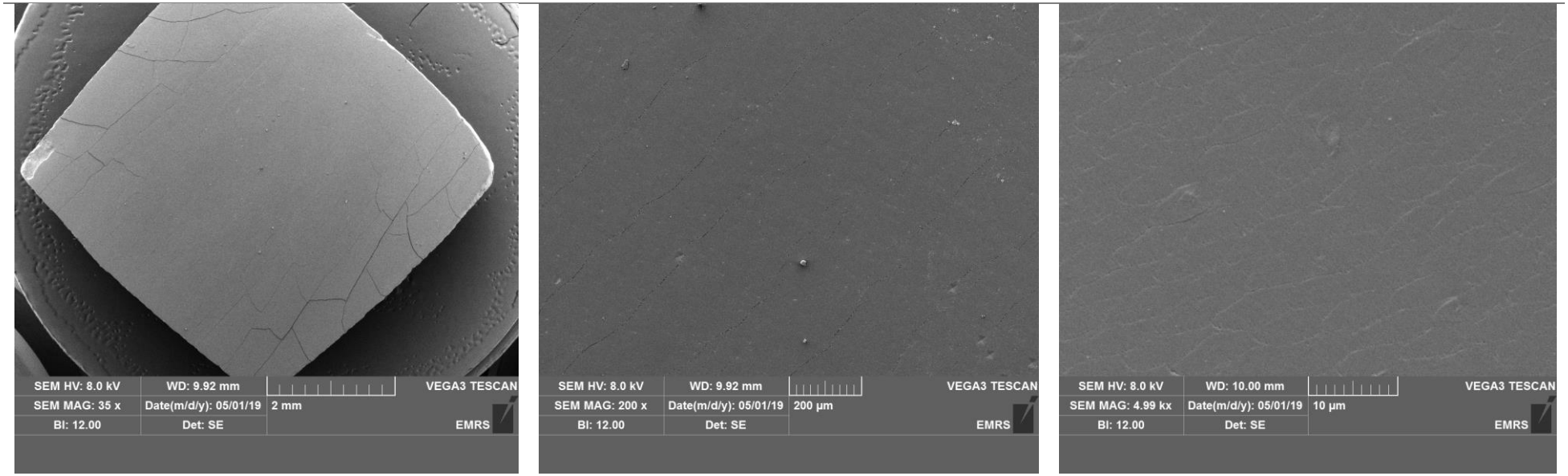


Figure 36 Three SEM images under low, medium, and high magnification of enamel treated with pH7 6% HP. Note the overall smooth surface with visible cracks under low magnification

pH9

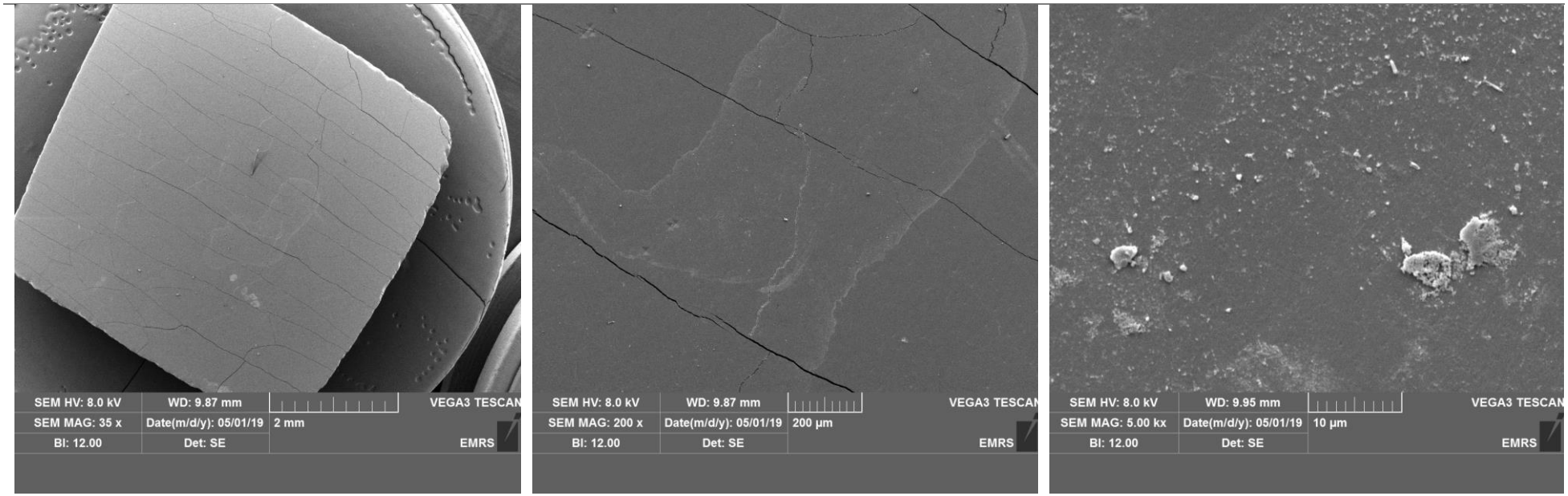


Figure 37 Three SEM images under low, medium, and high magnification of enamel treated with pH9 6% HP. Note the minor surface deformation under high magnification.

Control

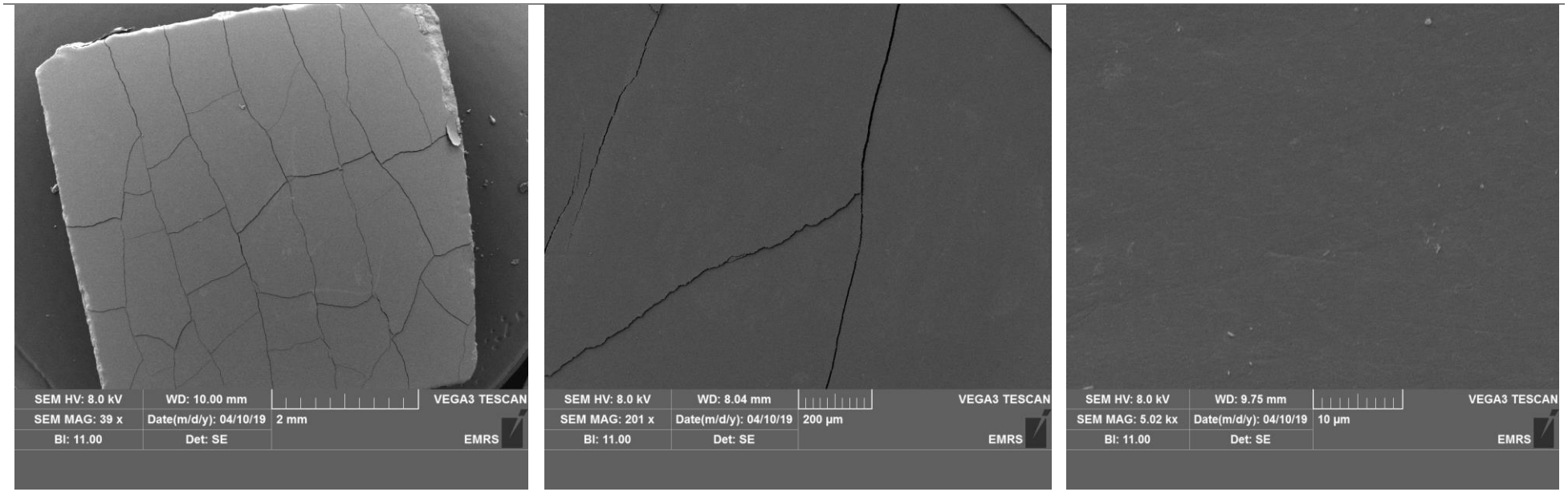


Figure 38 Three SEM images under low, medium, and high magnification of enamel treated with PBS. Note the overall smooth surface with visible cracks under low magnification

5.6.2 Whitening using 20% Hydrogen Peroxide

Enamel surface topography

Enamel treated with pH5 and pH7 HP showed minor surface irregularities in comparison to the control. The pH9 HP, on the other hand, caused significant surface irregularities and enamel deformation visible as sharp and narrow peaks of exposed enamel prisms separated by deep valleys (Figure 39).

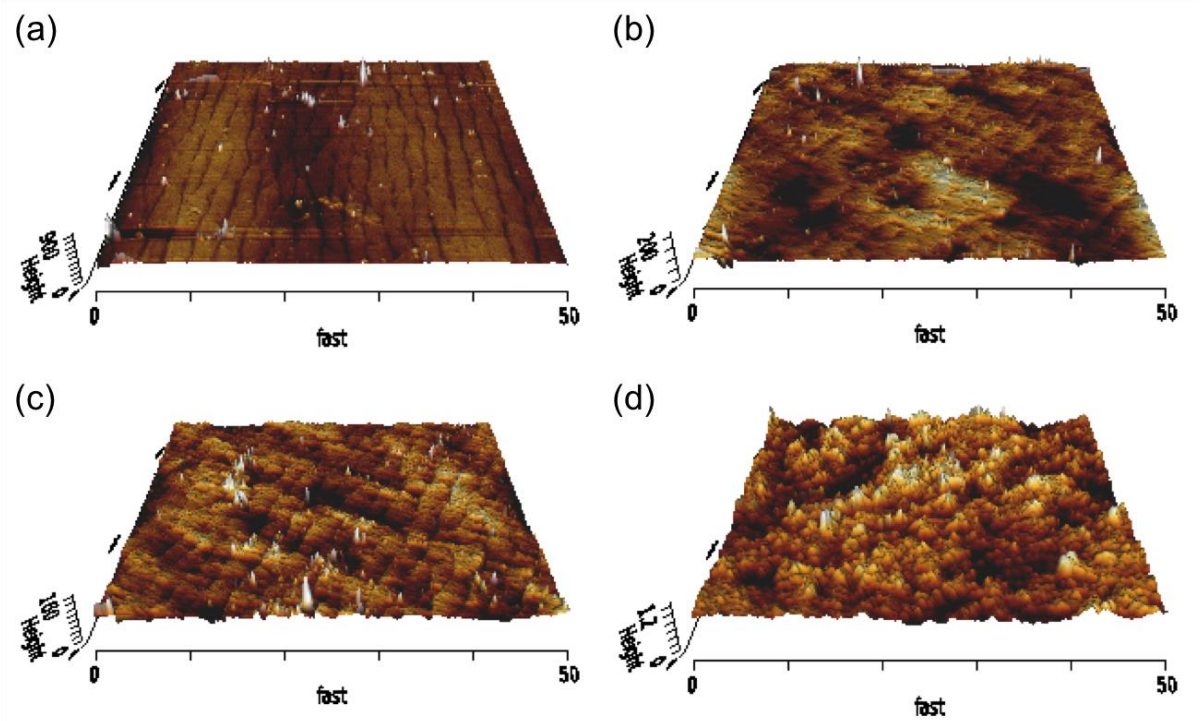


Figure 39 Representative 3D images of a control bovine enamel sample (a), treated with pH5 20% HP (b), treated with pH7 20% HP (c), and treated with pH9 20% HP (d). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-c) is nm and in image (d) is μm.

Enamel roughness (Ra)

After treatment with pH5 and pH9 HP, a significant increase in enamel Ra, by 25% and 78% respectively, was recorded (P≤0.05) (Table 18) (Figure 40). Treatment with pH7 HP, on the other hand, caused no significant changes in enamel roughness (P>0.05). Enamel samples treated with pH9 HP were significantly rougher than those treated with pH5 and pH7 HP in addition to the control enamel samples (P≤0.05).

	Ra (nm)	
	Before	After
pH5	5.6 (1.6)^{a,A}	7.0 (2.2)^{b,A}
pH7	6.4 (3.0)^{a,A}	6.9 (2.2)^{a,A}
pH9	6.8 (4.8)^{a,A}	12.1 (44.0)^{b,B}
Control	7.6 (2.8)^{a,A}	6.1 (2.0)^{a,A}

Table 18 Median (IQR) values of enamel Ra before and after treatment using pH5, pH7, and pH9 20% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

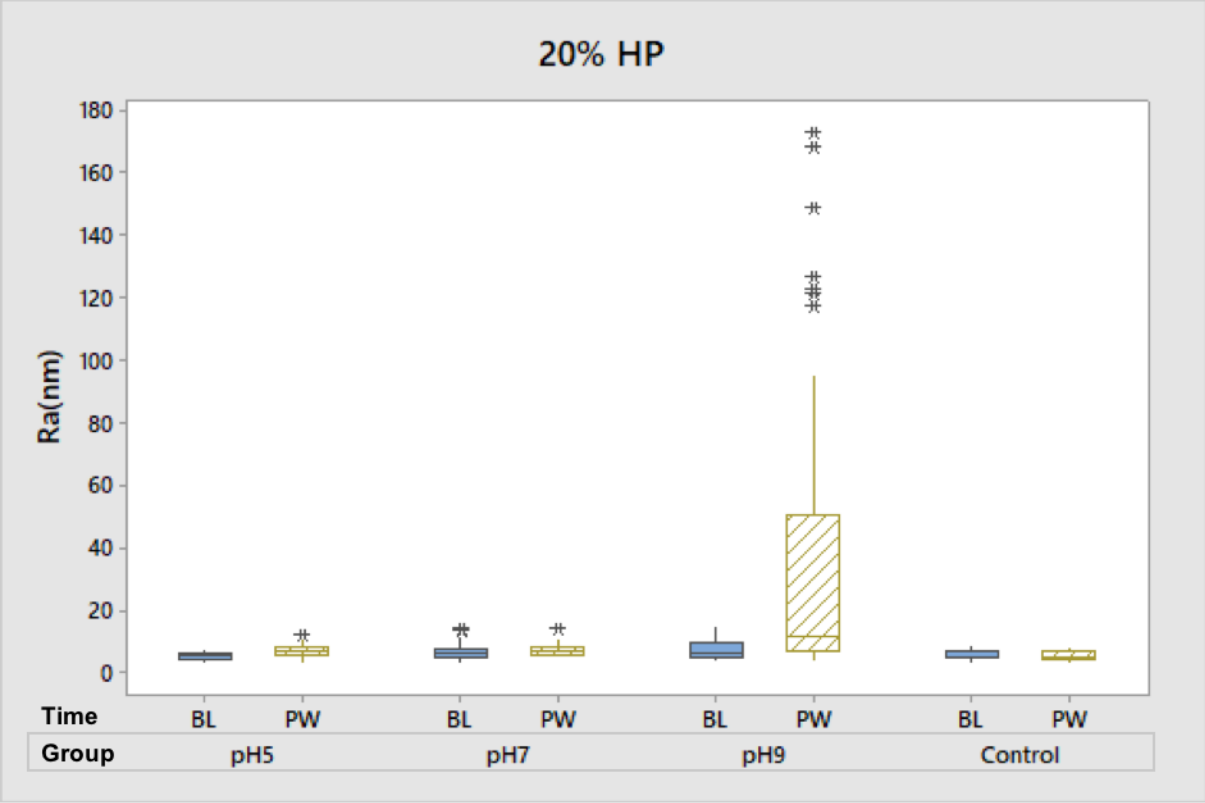


Figure 40 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 20% HP in addition to a control group. Note the great variability in enamel Ra after treatment with pH9 HP. Error bars represent the variability of data.

Enamel Hardness (HV)

Treatment with pH5, pH7, and pH9 HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 19) (Figure 41). The greatest decrease in hardness occurred in enamel treated with pH9 HP by 25%, followed by pH7 HP by 23%, and pH5 HP by 16%. After whitening treatments, enamel hardness did not significantly differ between treatment groups (P>0.05).

HV (H_{IT}) - 20%		
Treatment	Before	After
pH5	322.0 (55.7)^{a,A}	271.4 (43.4)^{b,A}
pH7	330.7 (25.4)^{a,A}	254.6 (41.7)^{b,A}
pH9	327.7 (48.0)^{a,A}	245.6 (81.3)^{b,A}
Control	323.1 (97.4)^{a,A}	256.1 (95.4)^{a,B}

Table 19 Median (IQR) values of enamel Vickers hardness before and after treatment using pH5, pH7, and pH9 20% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

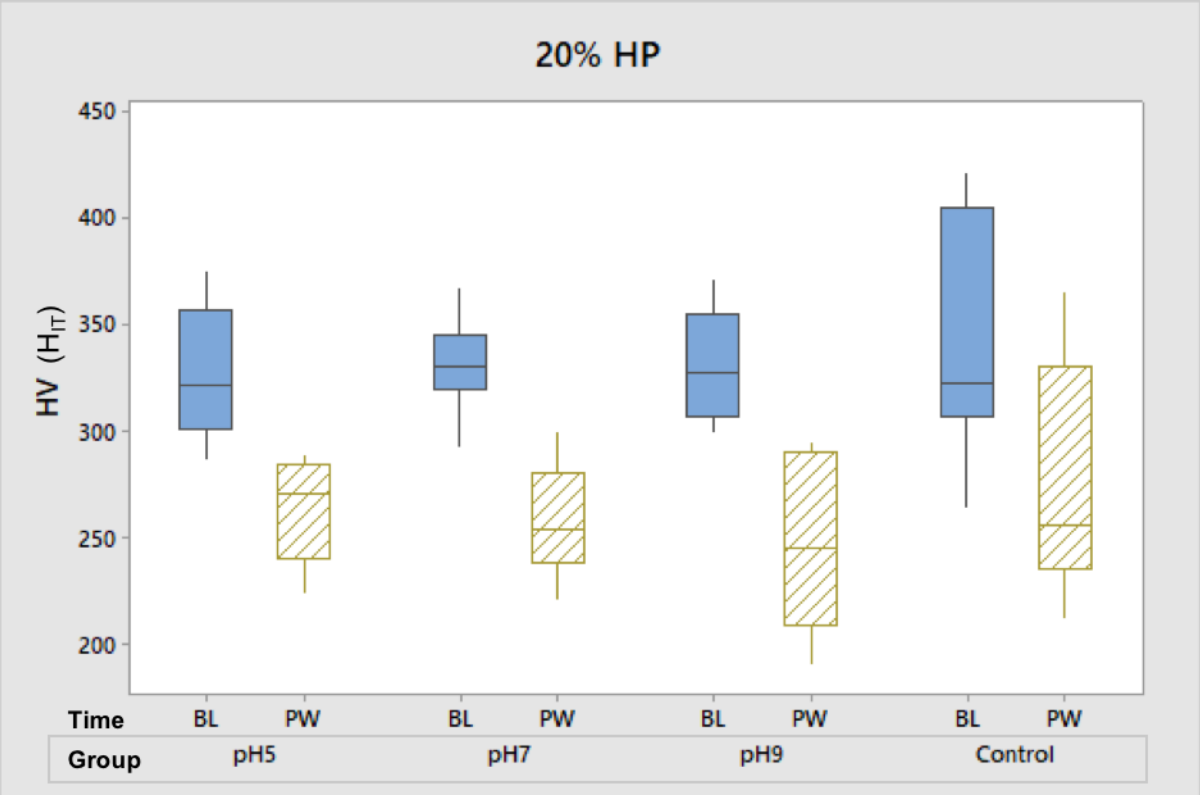


Figure 41 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 20% HP in addition to a control group. Note the HV reduction in all treatment groups with the greatest reduction in enamel treated with pH9 HP. Error bars represent the variability of data.

Colour change

The greatest colour change (ΔE) occurred in enamel treated with pH5 HP followed by pH7 HP, pH9 HP, and finally the control group; showing no significant changes in colour (ΔE≤2) (Table 20). Treatment with pH5, pH7, and pH9 HP caused a significant increase in enamel lightness (L*) and a significant decrease in (b*) values away from the yellow towards the blue spectrum (P≤0.05). After treatment with pH5 and pH9 HP, enamel samples showed a significant decrease in (a*) values towards the green spectrum (P≤0.05). Control enamel samples showed no significant changes in all colour parameters (L*), (a*), and (b*) (P>0.05). Changes in L*, a*, and b* values were not significantly different between whitened enamel samples (P>0.05).

ΔE			
pH5	pH7	pH9	Control
11.02 (0.9)[a]	9.1 (1.3)[a]	8.3 (1.9)[c]	1.6 (1.1)[d]
L* value			
	Before	After	
pH5	80.4 (1.3)[a,A]	86.8 (1.2)[b,A]	
pH7	81.3 (1.6)[a,A]	85.3 (0.9)[b,A]	
pH9	81.4 (1.2)[a,A]	86.4 (1.3)[b,A]	
Control	80.8 (1.3)[a,A]	82.2 (0.8)[a,B]	
a* value			
	Before	After	
pH5	-0.7 (0.3)[a,A]	-1.0 (0.2)[b,A]	
pH7	-0.8 (0.3)[a,A]	-1.0 (0.1)[a,A]	
pH9	-0.9 (0.3)[a,A]	-1.3 (0.2)[b,A]	
Control	-0.8 (0.4)[a,A]	-0.6 (0.5)[a,B]	
b* value			
	Before	After	
pH5	10.0 (0.8)[a,A]	1.1 (1.1)[b,A]	
pH7	8.2 (1.0)[a,A]	0.04 (0.2)[b,A]	
pH9	8.5 (2.3)[a,A]	1.9 (1.2)[b,A]	
Control	9.8 (1.0)[a,A]	11.7 (1.8)[a,B]	

Table 20 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH5, pH7, and pH9 20% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with pH5, pH7, and pH9 HP, atomic % of enamel calcium, phosphorus, and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 21).

Group	Element	Atomic %
pH5	Ca	26.0 (0.1)
	P	21.0 (0.2)
	C	13.1 (0.6)
pH7	Ca	25.9 (0.6)
	P	20.4 (0.4)
	C	13.2 (0.8)
pH9	Ca	26.4 (0.3)
	P	20.0 (0.3)
	C	13.5 (1.3)
Control	Ca	25.6 (1.2)
	P	20.4 (0.9)
	C	13.4 (0.5)

Table 21 Mean atomic % values and standard deviation (SD) of Calcium, Phosphorous, and Carbon in enamel treated using pH5, 7, and 9 of 20% HP in comparison to a control group.

Surface morphology

Enamel samples treated with pH7 HP have a smooth surface with no visible enamel damage as a result of the whitening process. After treatment with pH5 HP erosive damage was observed under high magnification through the development of surface pitting. The pH9 HP caused distinct surface changes in treated surfaces by the development of craters, pits, and the deformation of prismatic and inter-prismatic enamel (Figure 42-44).

pH5

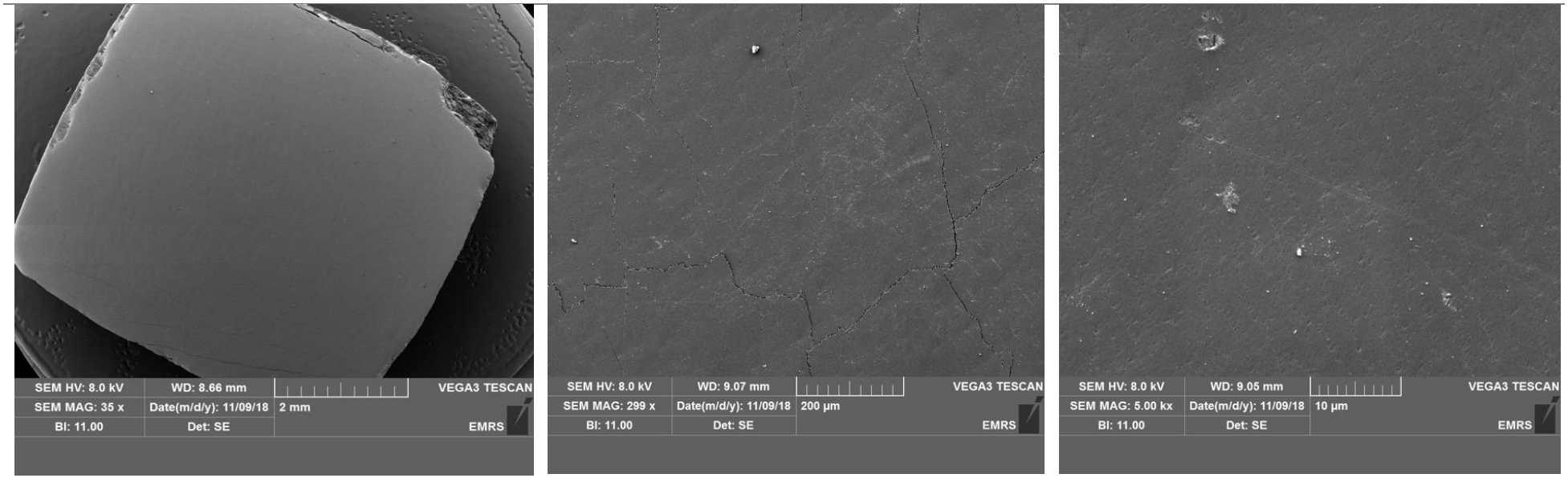


Figure 42 Three SEM images under low, medium, and high magnification of enamel treated with pH5 20% HP. Note the overall smooth surface with visible pitting in the enamel surface under high magnification.

pH7

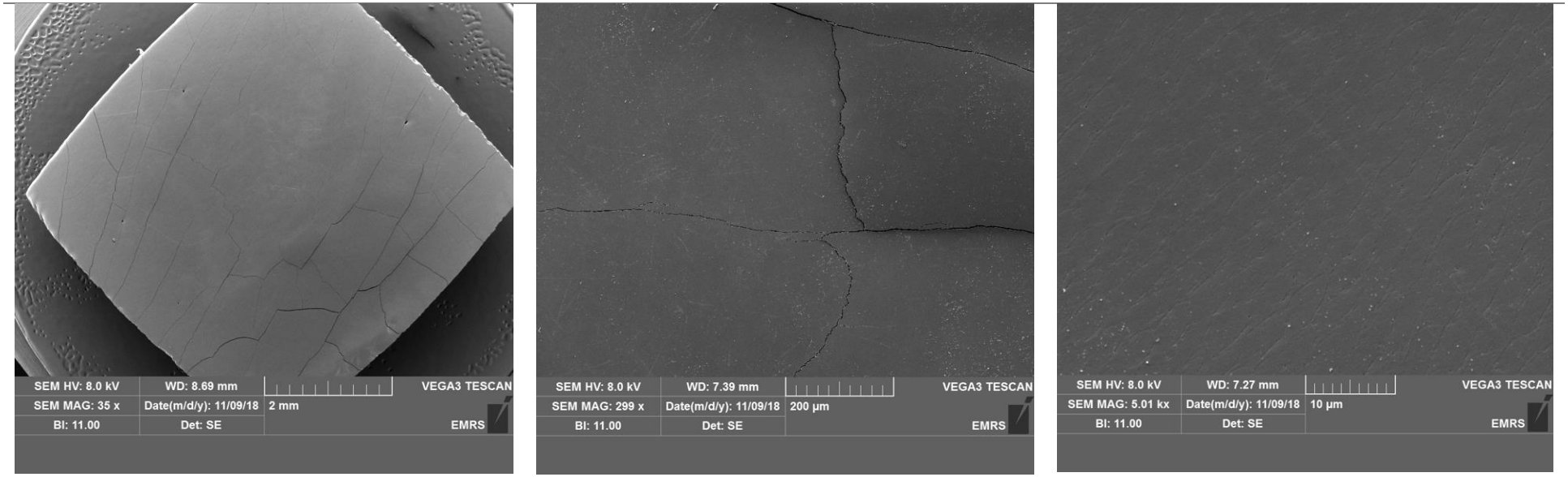


Figure 43 Three SEM images under low, medium, and high magnification of enamel treated with pH7 20% HP. Note the overall smooth surface with visible cracks under low magnification.

pH9

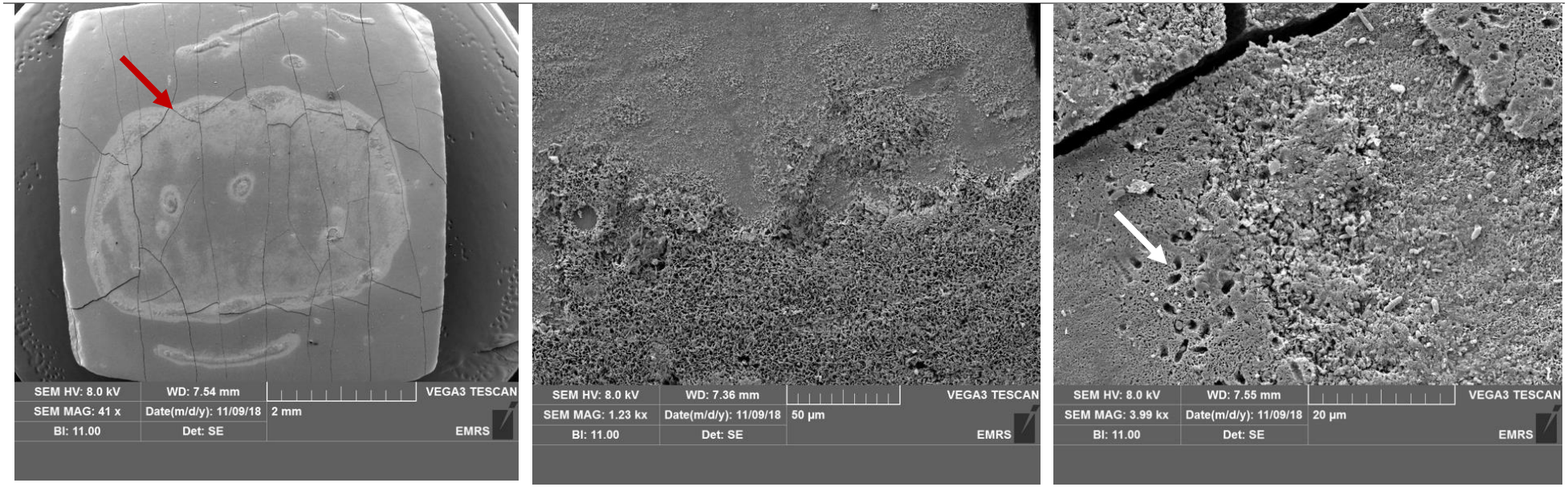


Figure 44 Three SEM images under low, medium, and high magnification of enamel treated with pH9 20% HP. Note the development of craters and the presence of deformed prismatic and inter-prismatic enamel under medium and high magnification. Under high magnification enamel pits are visible (white arrow), and under low magnification demineralisation of enamel can be clearly seen (red arrow).

5.6.3 Whitening using 40% Hydrogen Peroxide

Enamel surface topography

Treatment with pH5 HP caused enamel erosion. The organic matter; having a honeycomb outline, appeared lighter in colour which indicate a more elevated i.e. un-affected surface in comparison to the inorganic prisms which appeared darker in colour, indicating a more depressed i.e. eroded surface. Similarly, surface deformation appeared in enamel treated with pH7 HP and was less severe than that caused by the pH5 HP. Treatment with pH9 HP caused significant surface irregularities and deformation visible as round and blunt peaks separated by deep valleys (Figure 45).

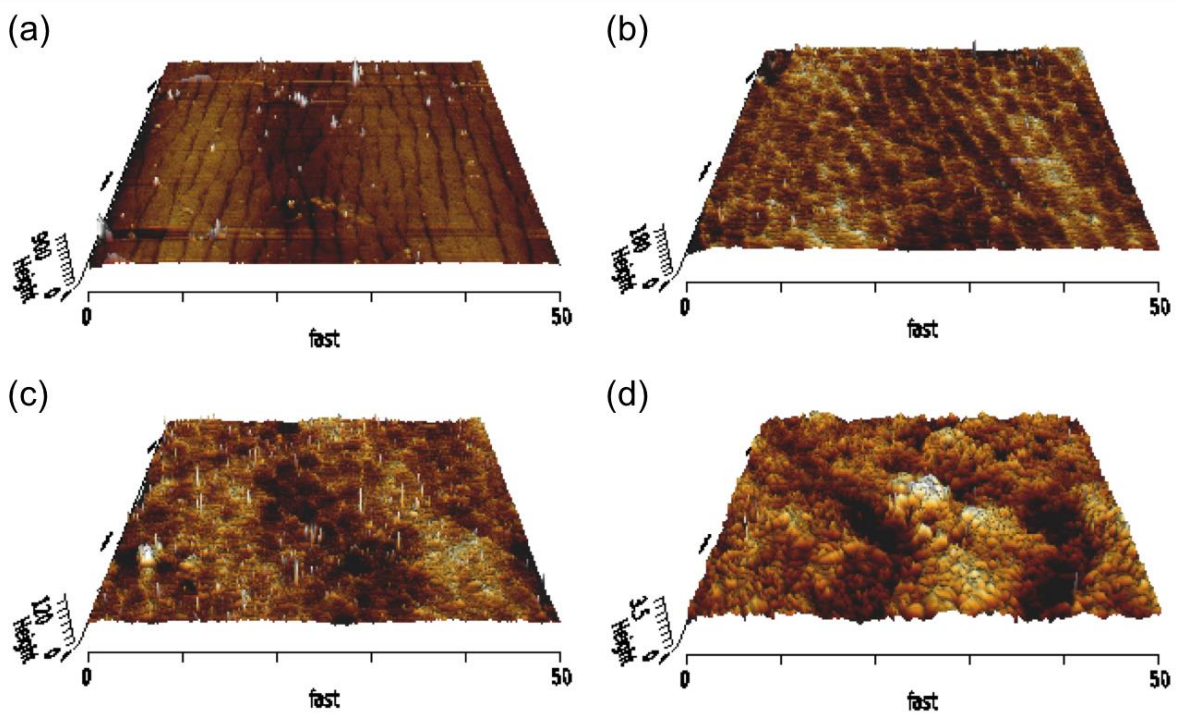


Figure 45 Representative 3D images of a control bovine enamel sample (a), treated with pH5 40% HP (b), treated with pH7 40% HP (c), and treated with pH9 40% HP (d). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-c) is nm and in image (d) is μm.

Enamel roughness (Ra)

Treatment with pH9 HP caused the greatest significant increase in enamel Ra, followed by treatment with pH7 HP (P≤0.05) (Table 22) (Figure 46). Enamel treated with pH5 HP, on the other hand, showed no significant changes in enamel Ra in comparison to BL. In addition, roughness values were not significantly different between enamel treated with pH5 HP and control enamel samples (P>0.05).

	Ra (nm)	
Treatment	Before	After
pH5	4.9 (2.3)^{a,A}	4.3 (1.3)^{a,A}
pH7	5.8 (4.1)^{a,A}	11.6 (7.6)^{b,B}
pH9	5.9 (2.9)^{a,A}	63.6 (217.5)^{b,C}
Control	7.6 (2.8)^{a,A}	6.1 (2.0)^{a,A}

Table 22 Median (IQR) values of enamel Ra before and after treatment using pH5, pH7, and pH9 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

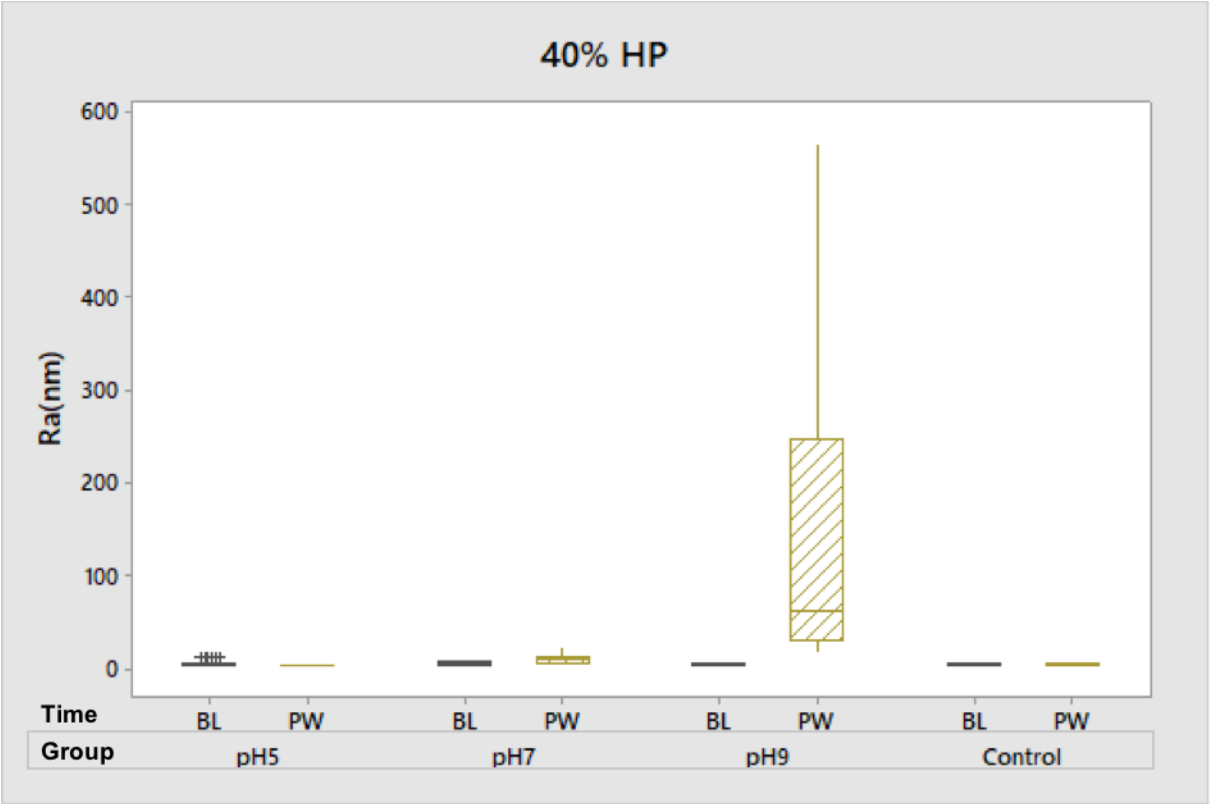


Figure 46 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 40% HP in addition to a control group. Note the great variability in enamel Ra after treatment with pH9 HP. Error bars represent the variability of data.

Enamel Hardness (HV)

Treatment with pH5, pH7, and pH9 HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 23) (Figure 47). Greatest reduction in hardness occurred in enamel treated with pH9 HP by 63%, followed by pH5 HP by 56%, and pH7 HP by 24%. Enamel hardness did not significantly differ between enamel treated with pH5 HP and pH9 HP (P>0.05).

	HV (H_{IT})	
Treatment	Before	After
pH5	335.0 (62.4)^{a,A}	148.2 (57.4)^{b,A}
pH7	327.6 (58.2)^{a,A}	248.5 (80.2)^{b,B}
pH9	315.7 (23.4)^{a,A}	118.2 (55.2)^{b,A}
Control	323.1 (97.4)^{a,A}	256.1 (95.4)^{a,B}

Table 23 Median (IQR) values of enamel Vickers hardness before and after treatment using pH5, pH7, and pH9 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

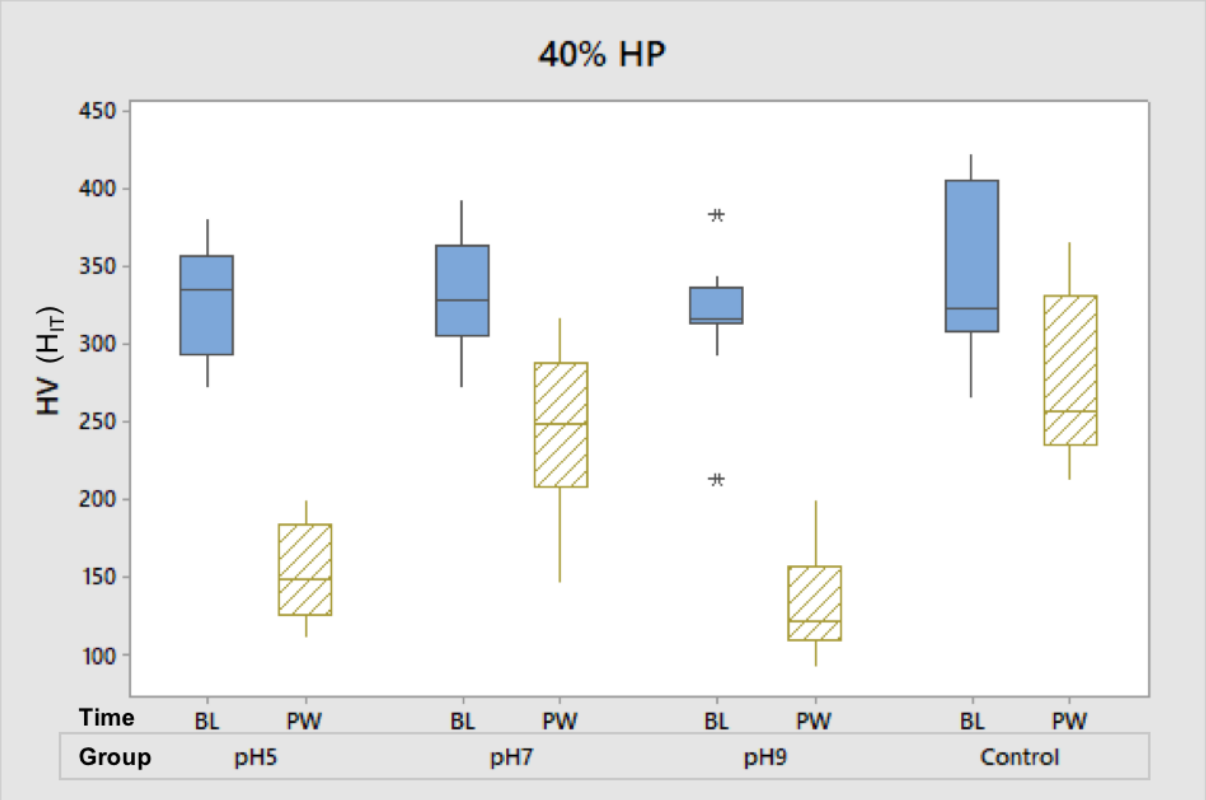


Figure 47 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH5, pH7, and pH9 40% HP in addition to a control group. Note the HV reduction in all treatment groups with the greatest reduction in enamel treated with pH9 HP. Error bars represent the variability of data.

Colour change

The greatest colour change (ΔE) occurred in enamel treated with pH5 HP followed by pH7 HP, pH9 HP, and finally the control group; showing no significant changes in colour (ΔE≤2). Treatment with pH5, pH7, and pH9 HP caused a significant increase in enamel lightness (L*) and a significant decrease in (b*) values away from the yellow towards the blue spectrum (P≤0.05) (Table 24). After treatment with pH5 and pH9 HP, enamel samples showed a significant decrease in (a*) values towards the green spectrum (P≤0.05). Control enamel samples showed no significant changes in all colour parameters (L*), (a*), and (b*) (P>0.05). Changes in L*, a*, and b* values were not significantly different between whitened enamel samples (P>0.05).

ΔE			
pH5	pH7	pH9	Control
13.0 (0.9)^{a}	11.2 (1.9)^{a,b}	9.5 (2.4)^{b}	1.6 (1.1)^{c}
L* value			
	Before	After	
pH5	80.7 (0.6)^{a,A}	89.7 (0.5)^{b,A}	
pH7	80.6 (1.6)^{a,A}	89.2 (1.2)^{b,A}	
pH9	82.0 (2.3)^{a,A}	89.2 (0.7)^{b,A}	
Control	80.8 (1.3)^{a,A}	82.2 (0.8)^{a,B}	
a* value			
	Before	After	
pH5	-0.6 (0.2)^{a,A}	-1.3 (0.1)^{b,A}	
pH7	-1.4 (0.2)^{a,A}	-1.3 (0.04)^{a,A}	
pH9	-0.7 (0.5)^{a,A}	-1.5 (0.1)^{b,A}	
Control	-0.8 (0.4)^{a,A}	-0.6 (0.5)^{a,B}	
b* value			
	Before	After	
pH5	10.6 (1.8)^{a,A}	1.4 (0.9)^{b,A}	
pH7	8.0 (1.5)^{a,A}	1.0 (0.6)^{b,A}	
pH9	8.5 (1.6)^{a,A}	2.6 (0.7)^{b,A}	
Control	9.8 (1.0)^{a,A}	11.7 (1.8)^{a,B}	

Table 24 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH5, pH7, and pH9 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with pH5, pH7, and pH9 HP, atomic % of enamel calcium and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 25). The atomic % of enamel phosphorus was significantly greater in whitened enamel samples than control samples (P≤0.05).

Group	Element	Atomic %
pH5	Ca	28.9 (1.2)
	P	26.0 (0.0)
	C	7.7 (1.6)
pH7	Ca	28.2 (5.0)
	P	25.3 (0.8)
	C	5.7 (8.1)
pH9	Ca	25.4 (1.2)
	P	24.6 (0.7)
	C	8.7 (2.8)
Control	Ca	25.6 (1.2)
	P	20.4 (0.9)
	C	13.4 (0.5)

Table 25 Mean atomic % values and standard deviation (SD) of enamel surface Calcium, Phosphorous, and Carbon after whitening using pH5, pH7, and pH9 of 40% HP in comparison to a control group.

Surface morphology

Enamel deformation increased as pH values increased. Surface erosion appeared in enamel treated with pH5 HP; visible as localised surface pitting. The development of craters, deformation of prismatic/inter-prismatic structure, and the formation of grooves occurred in enamel treated with pH7 and pH9 HP (Figure 48-50).

pH5

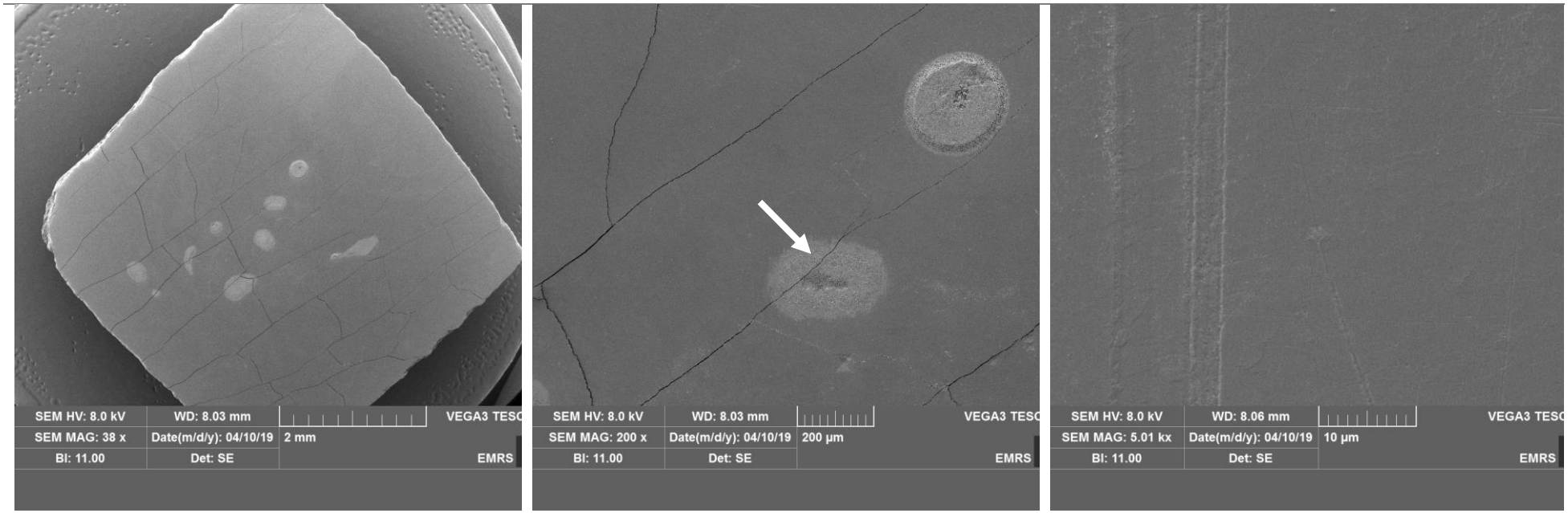


Figure 48 Three SEM images under low, medium, and high magnification of enamel treated with pH5 40% HP. Note the overall smooth surface with visible areas of erosion (arrow).

pH7

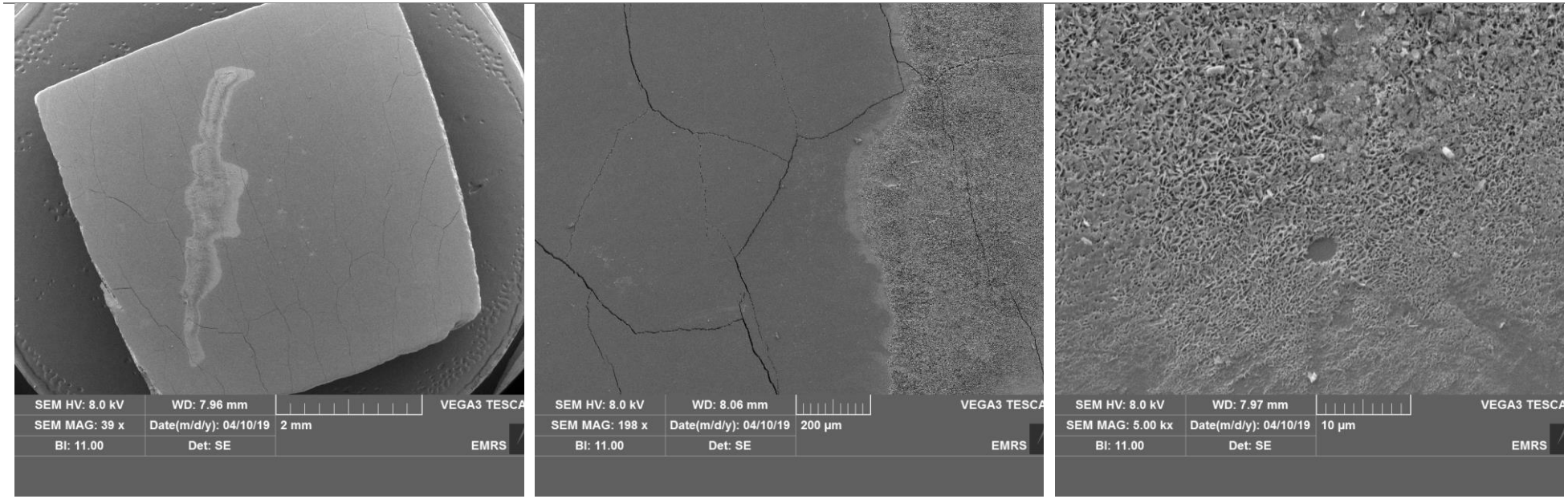


Figure 49 Three SEM images under low, medium, and high magnification of enamel treated with pH7 40% HP. Note the overall smooth surface with visible localised enamel deformation.

pH9

Figure 50 Three SEM images under low, medium, and high magnification of enamel treated with pH9 40% HP. Note enamel deformation by the development of pits, craters (arrow), and grooves under high magnification.

5.6.4 Whitening using acidic hydrogen peroxide

Enamel surface topography

After treatment with HP, enamel appeared smooth and undamaged; similar to the control enamel sample (Figure 51). Differences are difficult to see due to the differences in z-scale in the images.

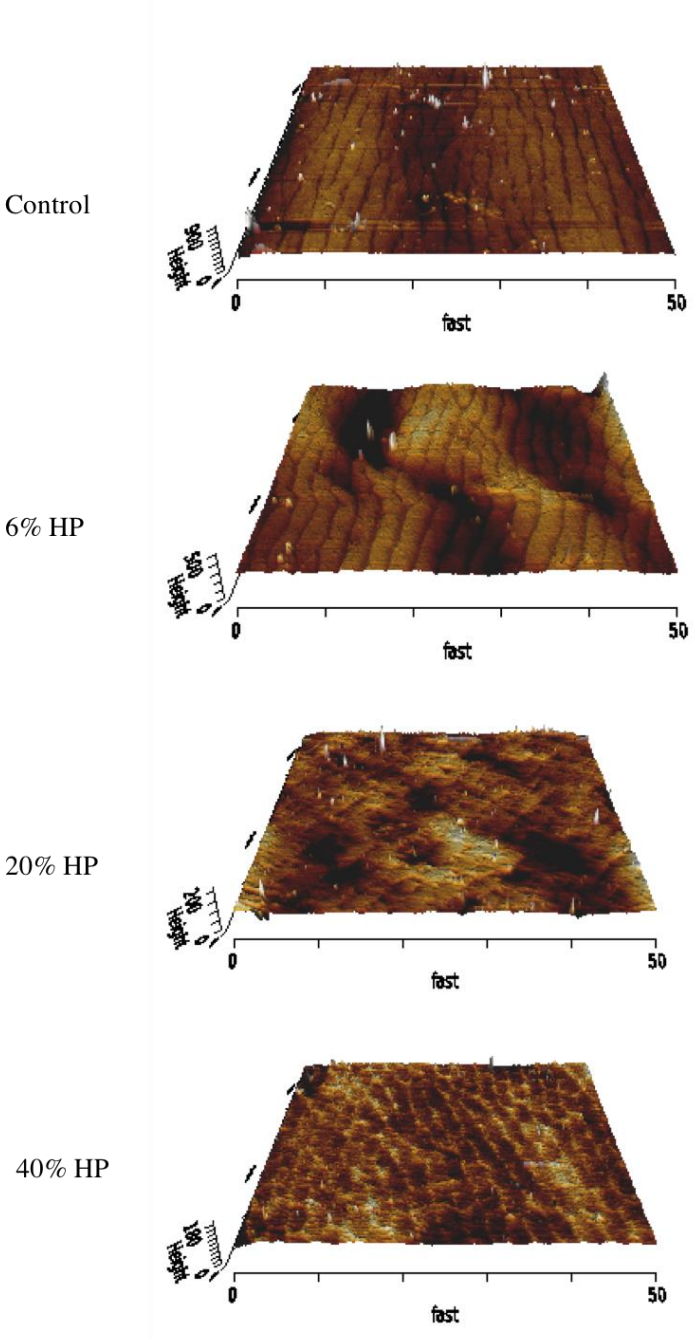


Figure 51 Representative 3D images of a control bovine enamel sample, treated with pH5 6% HP, treated with pH5 20% HP, and treated with pH5 40% HP. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed.

Enamel Roughness (Ra)

Treatment with 6% and 40% HP caused no significant changes in enamel Ra (P>0.05). The 20% HP, on the other hand, caused a significant increase in Ra values (P≤0.05). After treatment with 6%, 20%, and 40% HP, recorded Ra values were significantly different between treatment groups (P≤0.05); with highest recorded Ra in enamel treated with 20% HP and lowest values recorded in enamel treated with 40% HP (Table 26) (Figure 52).

Ra (nm) - pH5		
	Before	After
6%	6.1 (5.6)^{a,A}	5.1 (3.8)^{a,A}
20%	5.6 (1.6)^{a,A}	7.0 (2.2)^{b,B}
40%	4.9 (2.3)^{a,A}	4.3 (1.3)^{a,C}
Control	7.6 (2.8)^{a,A}	6.1 (2.0)^{a,A,B}

Table 26 Median (IQR) values of enamel Ra before and after treatment using pH5 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

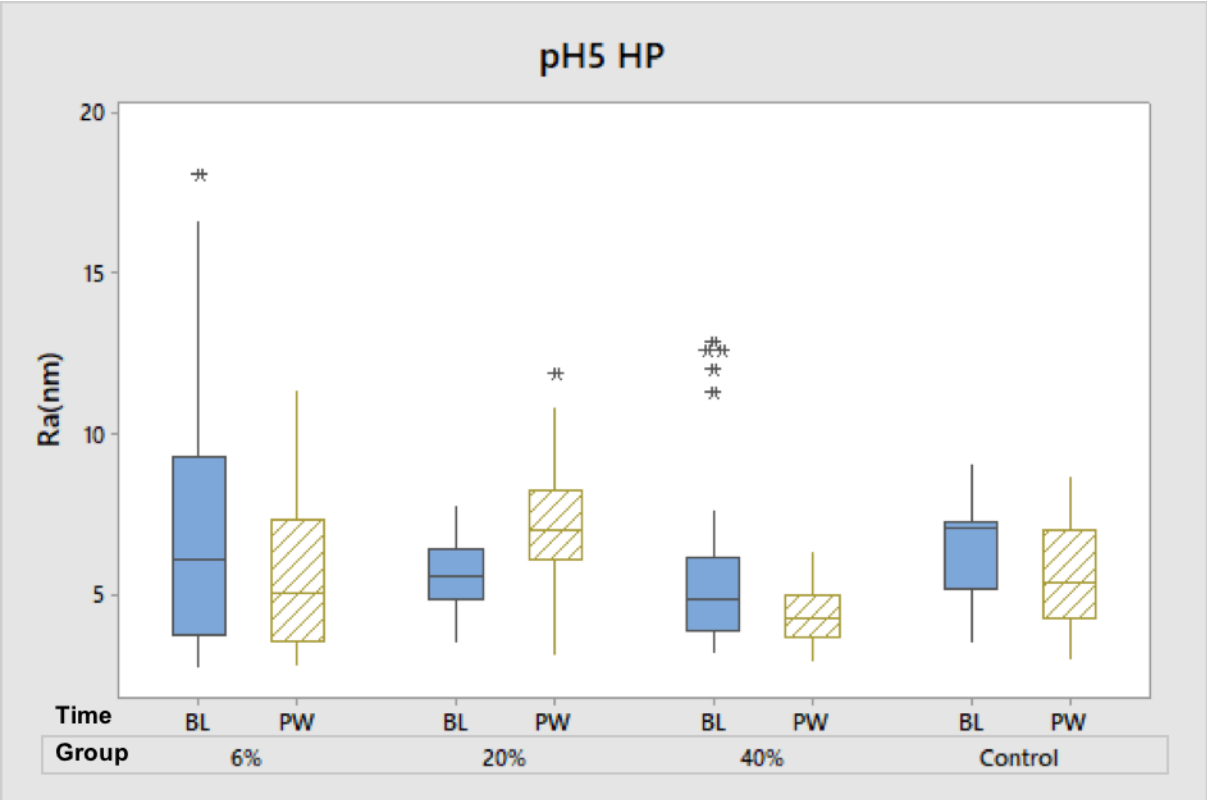


Figure 52 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH5 6%, 20%, and 40% HP in addition to a control group. Error bars represent the variability of data.

Enamel Hardness (HV)

Treatment with 6%, 20%, and 40% HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 27) (Figure 53). The greatest hardness reduction occurred in enamel treated with 40% HP (P≤0.05), which was significantly lower than HV values recorded after treatment with 6% and 20% HP in addition to the control group (P≤0.05).

Hardness (HV) - pH5		
	Before	After
6%	336.0 (21.4)^{a,A}	271.7(83.5)^{b,A}
20%	322.0 (55.7)^{a,A}	271.4(43.4)^{b,A}
40%	335.0 (62.4)^{a,A}	148.2(57.4)^{b,B}
Control	323.1 (97.4)^{a,A}	256.1(95.4)^{a,A}

Table 27 Median (IQR) values of enamel Vickers hardness before and after treatment using pH5 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

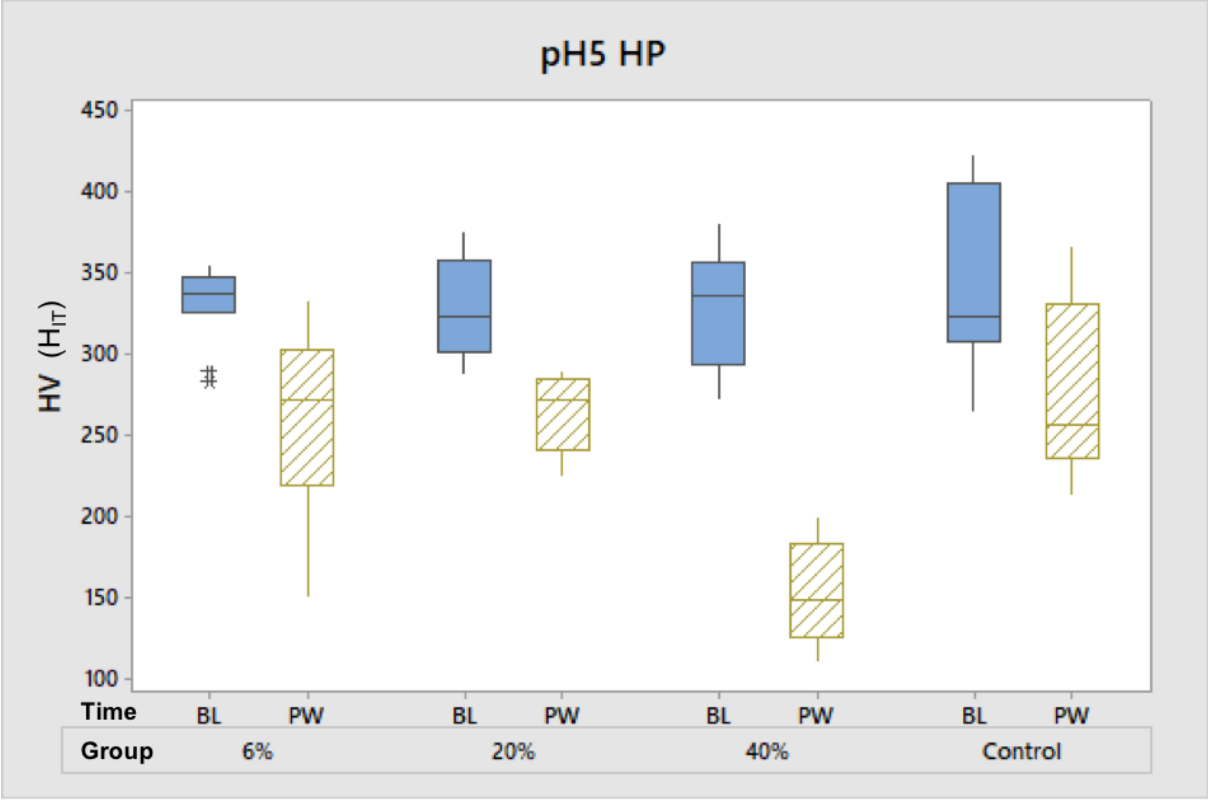


Figure 53 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH5 6%, 20%, and 40% HP in addition to a control group. Error bars represent the variability of data.

Colour change

The greatest colour change (ΔE) occurred in enamel treated with 40% HP followed by 20% HP, 6% HP, and finally the control group; showing no significant changes in colour (ΔE≤2) (Table 28). A significant increase in enamel lightness (L*) and decrease in (a*) values towards the green spectrum and (b*) values away from the yellow towards the blue spectrum occurred in enamel whitened using 6%, 20%, and 40% HP (P≤0.05) (Figure 54). Enamel lightness was significantly greater after treatment with 40% HP in comparison to samples treated with 20% and 6% HP (P≤0.05).

ΔE			
6%	20%	40%	Control
9.5 (1.5)^{a}	11.02 (0.9)^{a,b}	13.0 (0.9)^{b}	1.6 (1.1)^{c}
L* value			
	Before	After	
6%	83.0 (1.7)^{a,A}	85.9 (1.0)^{b,A}	
20%	80.4 (1.3)^{a,A}	86.8 (1.2)^{b,A}	
40%	80.7 (0.6)^{a,A}	89.7 (0.5)^{b,B}	
Control	80.8 (1.3)^{a,A}	82.2 (0.8)^{a,C}	
a* value			
	Before	After	
6%	-0.8 (0.3)^{a,A}	-1.4 (0.2)^{b,A}	
20%	-0.7 (0.3)^{a,A}	-1.0 (0.2)^{b,A}	
40%	-0.6 (0.2)^{a,A}	-1.3 (0.1)^{b,A}	
Control	-0.8 (0.4)^{a,A}	-0.6 (0.5)^{a,B}	
b* value			
	Before	After	
6%	8.9 (1.5)^{a,A}	-0.1 (0.2)^{b,A}	
20%	10.0 (0.8)^{a,A}	1.1 (1.1)^{b,A}	
40%	10.6 (1.8)^{a,A}	1.4 (0.9)^{b,A}	
Control	9.8 (1.0)^{a,A}	11.7 (1.8)^{a,B}	

Table 28 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH5 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

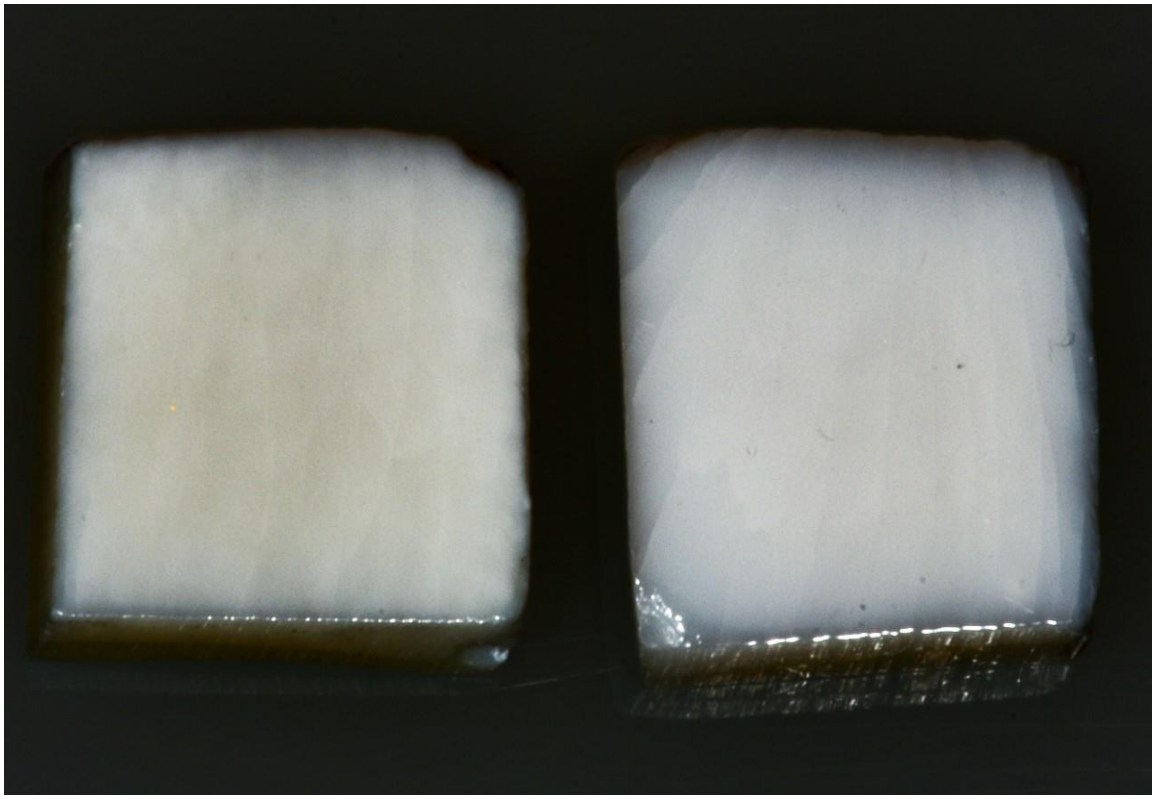


Figure 54 Bovine enamel whitened using pH5 20% HP (right) in comparison to a control un-treated bovine enamel sample (left). Note the lighter enamel sample after treatment with HP and the reduction in b value i.e. decrease in sample yellowness clearly visibly after whitening.*

Mineral composition

After treatment with 6%, 20%, and 40% HP, atomic % of calcium and carbon were not significantly different than the those recorded in the control group ($P>0.05$). Treatment with 40% HP, however, caused a significant increase in enamel phosphorus in comparison to other treatment groups ($P\leq0.05$) (Table 29).

	Element	Atomic %
6%	Ca	27.0 (0.8)^{A}
	P	19.9 (0.1)^{A}
	C	13.8 (0.4)^{A}
20%	Ca	26.0 (0.1)^{A}
	P	21.0 (0.2)^{A}
	C	13.0 (0.6)^{A}
40%	Ca	28.9 (1.2)^{A}
	P	26.0 (0.0)^{B}
	C	7.7 (1.6)^{A}
Control	Ca	25.6 (1.2)^{A}
	P	20.4 (0.9)^{A}
	C	13.4 (0.5)^{A}

Table 29 Mean atomic % values and standard deviation (SD) of enamel Calcium, Phosphorous, and Carbon after whitening using pH5 6%, 20%, and 40% HP in comparison to a control group. Different superscript letters indicate significant differences between groups.

Surface morphology

Enamel treated with 6% HP had an overall smooth surface with no visible damage (Figure 55-57). After treatment with 20% HP erosive damage was observed under high magnification through the development of surface pitting. More severe surface erosion appeared in enamel treated with 40% HP.

6% HP

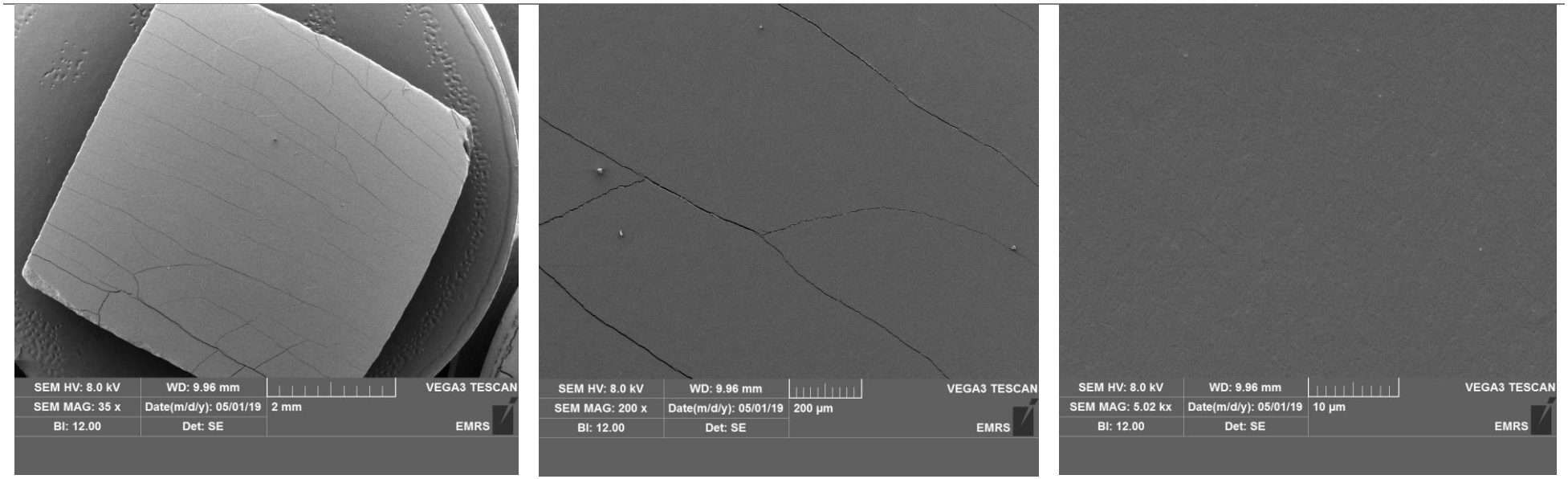


Figure 55 Three SEM images under low, medium, and high magnification of enamel treated with pH5 6% HP. Note the overall smooth surface with visible cracks under low magnification.

20% HP

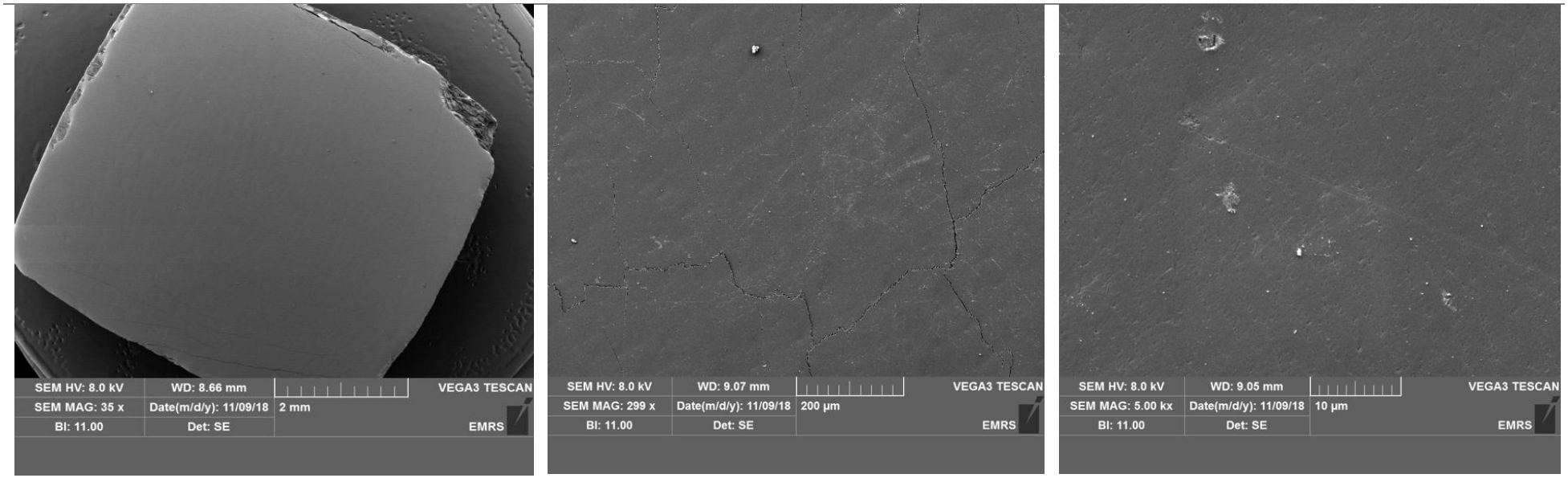


Figure 56 Three SEM images under low, medium, and high magnification of enamel treated with pH5 20% HP. Note the overall smooth surface with visible pitting in the enamel surface under high magnification.

40% HP

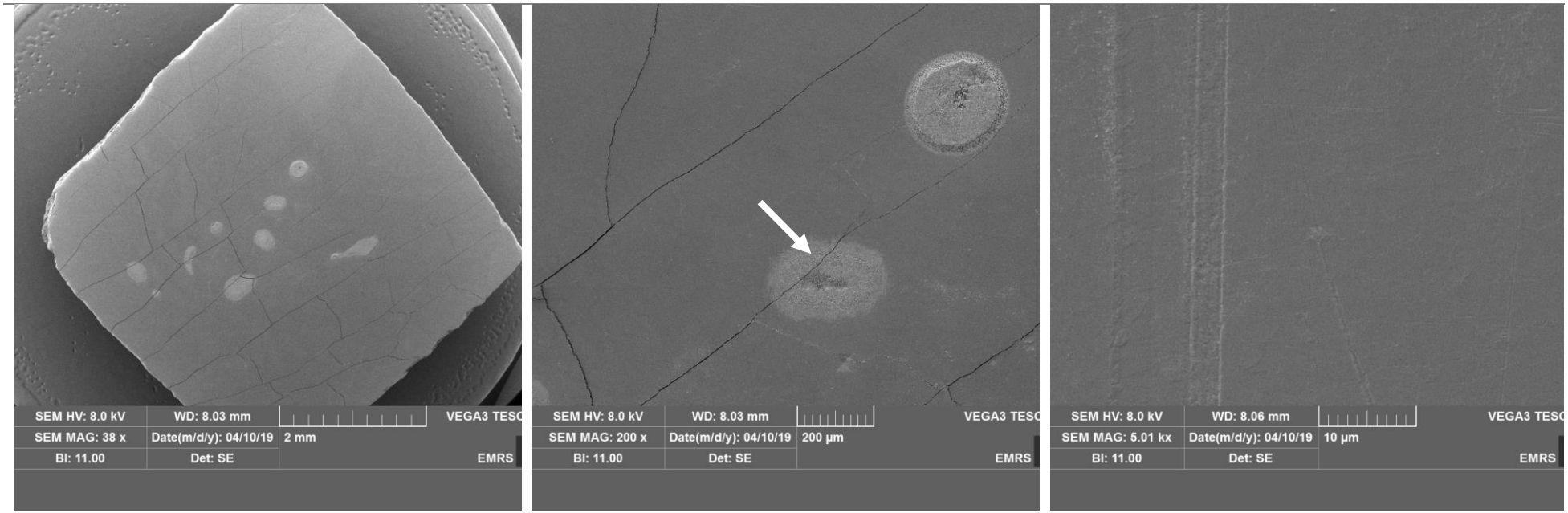


Figure 57 Three SEM images under low, medium, and high magnification of enamel treated with pH5 40% HP. Note the overall smooth surface with visible areas of erosion (arrow).

5.6.5 Whitening using neutral hydrogen peroxide

Enamel surface topography

After treatment with HP, enamel appeared smooth and undamaged; similar to the control enamel sample (Figure 58). Differences are difficult to see due to the differences in z-scale in images.

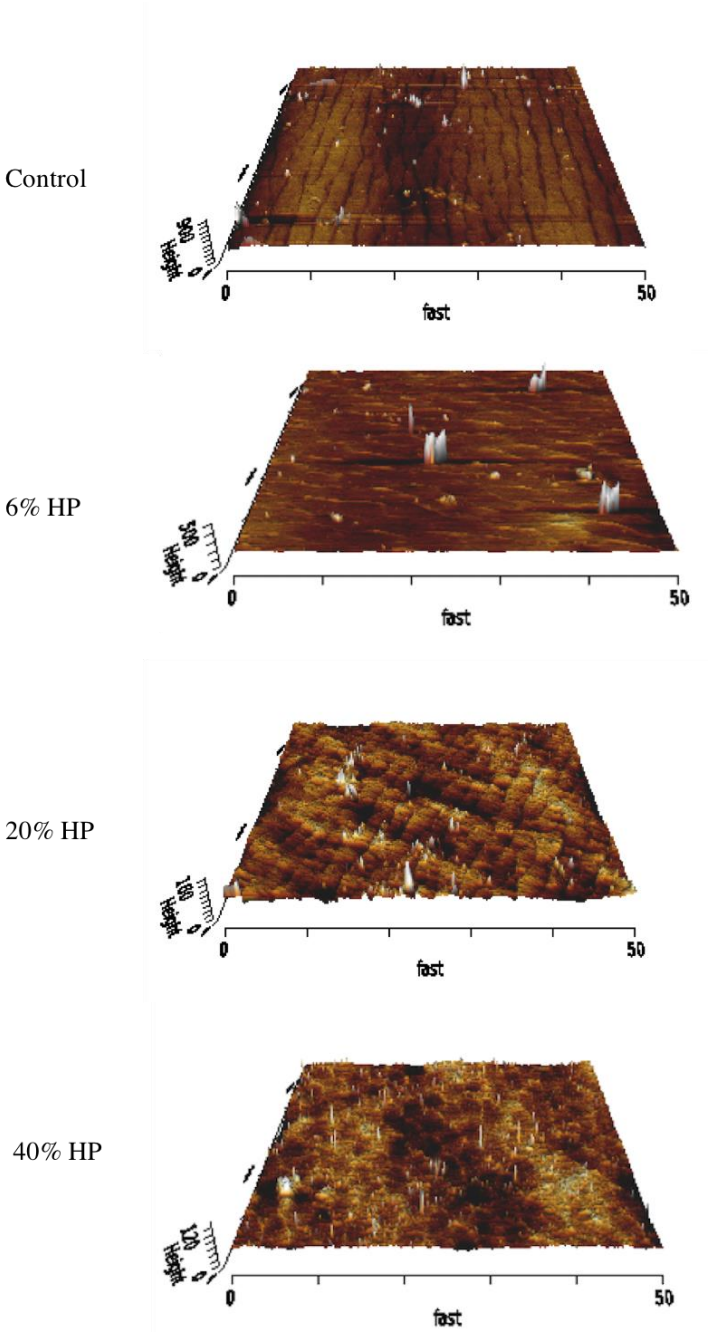


Figure 58 Representative 3D images of a control bovine enamel sample, treated with pH7 6% HP, treated with pH7 20% HP, and treated with pH7 40% HP. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed.

Enamel roughness (Ra)

Treatment with 6% and 20% HP caused no significant changes in enamel Ra (P>0.05). The 40% HP, on the other hand, caused a significant increase in enamel roughness which was significantly greater than roughness values recorded after treatment with 20% HP (P≤0.05) (Table 30) (Figure 59).

Ra (nm) – pH7		
	Before	After
6%	7.8(2.4)^{a,A}	8.6(5.3)^{a,A,C}
20%	6.4(3.0)^{a,A}	6.9(2.2)^{a,A}
40%	5.8(4.1)^{a,A}	11.6(7.6)^{b,C}
Control	7.6(2.8)^{a,A}	6.1(2.0)^{a,A}

Table 30 Median (IQR) values of enamel Ra before and after treatment using pH7 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

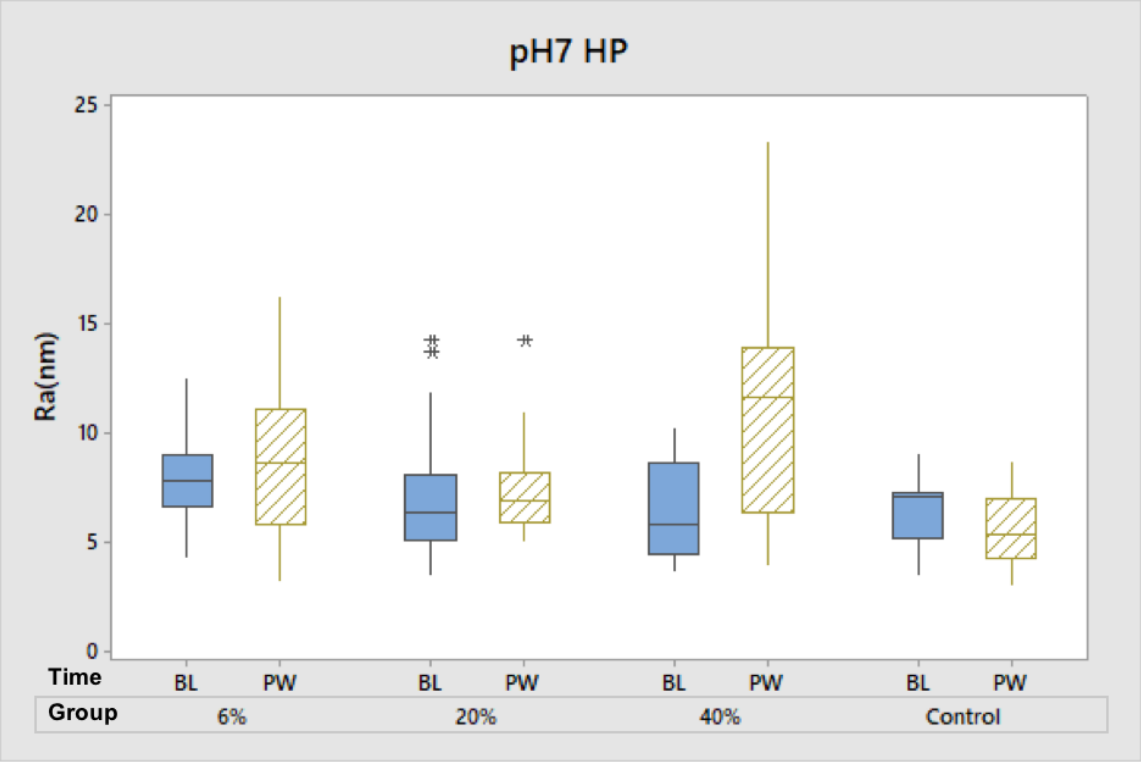


Figure 59 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH7 6%, 20%, and 40% HP in addition to a control group. Error bars represent the variability of data.

Enamel hardness (HV)

Treatment with 6%, 20%, and 40% HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 31) (Figure 60). After whitening, enamel hardness did not significantly differ between treatment groups (P>0.05).

Hardness (HV) – pH7		
	Before	After
6%	329.3 (42.6)^{a,A}	253.4 (78.9)^{b,A}
20%	330.7 (25.4)^{a,A}	254.6 (41.7)^{b,A}
40%	327.6 (58.2)^{a,A}	248.5 (80.2)^{b,A}
Control	323.1 (97.4)^{a,A}	256.1 (95.4)^{a,A}

Table 31 Median (IQR) values of enamel Vickers hardness before and after treatment using pH7 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

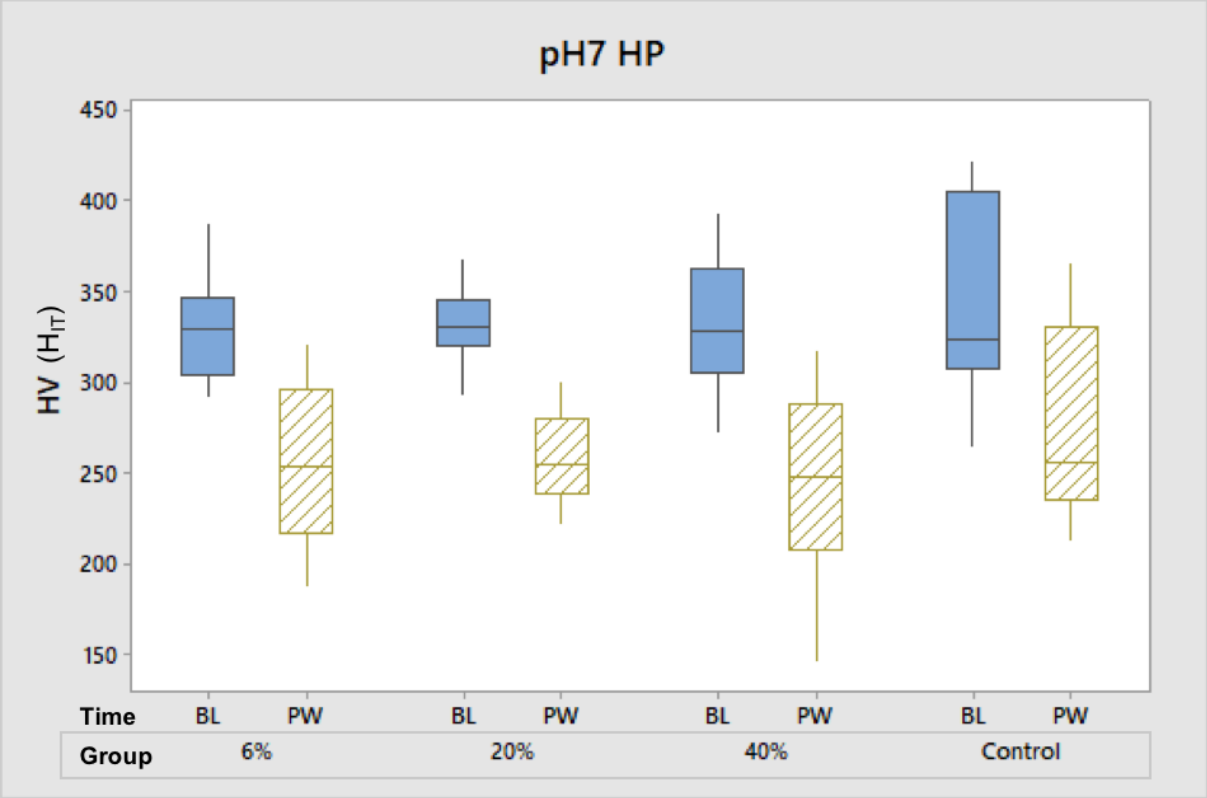


Figure 60 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH7 6%, 20%, 40% HP in addition to a control group. Error bars represent the variability of data.

Colour change

The greatest colour change (ΔE) occurred in enamel treated with 40% HP followed by 20% HP, 6% HP, and finally the control group; showing no significant changes in colour (ΔE≤2) (Table 32). A significant increase in lightness (L*) occurred in enamel samples treated with 20% and 40% HP (P≤0.05). Lightness values were significantly greater in enamel treated with 40% HP in comparison to other treatment groups (P≤0.05). A significant decrease in (a*) values towards the green spectrum only occurred in enamel treated with 6% HP (P≤0.05). The decrease in (b*) values away from the yellow towards the blue spectrum, on the other hand, occurred in enamel whitened using 6%, 20%, and 40% HP (P≤0.05). Changes in (b*) values were not significantly different between whitened enamel samples (P>0.05).

ΔE			
6%	20%	40%	Control
8.4 (1.7)^{a}	9.1 (1.3)^{a,b}	11.2 (1.9)^{b}	1.6 (1.1)^{c}
L*			
	Before	After	
6%	83.3 (1.4)^{a,A}	84.9 (1.5)^{a,A}	
20%	81.3 (1.6)^{a,A}	85.3 (0.9)^{b,A}	
40%	80.6 (1.6)^{a,A}	89.2 (1.2)^{b,B}	
Control	80.8 (1.3)^{a,A}	82.2 (0.8)^{a,C}	
a*			
	Before	After	
6%	-0.7 (0.2)^{a,A}	-1.4 (0.1)^{b,A}	
20%	-0.8 (0.3)^{a,A}	-1.0 (0.1)^{a,B}	
40%	-1.4 (0.2)^{a,A}	-1.3 (0.04)^{a,A}	
Control	-0.8 (0.4)^{a,A}	-0.6 (0.5)^{a,C}	
b*			
	Before	After	
6%	8.6 (1.1)^{a,A}	0.5 (0.5)^{b,A}	
20%	8.2 (1.0)^{a,A}	0.04 (0.2)^{b,A}	
40%	8.0 (1.5)^{a,A}	1.0 (0.6)^{b,A}	
Control	9.8 (1.0)^{a,A}	11.7 (1.8)^{a,B}	

Table 32 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH7 6%, 20%, and 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with 6%, 20%, and 40% HP, atomic % of calcium and carbon were not significantly different than the those recorded in the control group ($P>0.05$). Treatment with 40% HP, however, caused a significant increase in enamel phosphorus in comparison to other treatment groups ($P\leq0.05$) (Table 33).

	Element	Atomic %
6%	Ca	28.5 (0.3)^{A}
	P	20.4 (0.2)^{A}
	C	12.7 (0.1)^{A}
20%	Ca	25.9 (0.6)^{A}
	P	20.4 (0.4)^{A}
	C	13.2 (0.8)^{A}
40%	Ca	28.2 (5.0)^{A}
	P	25.3 (0.8)^{B}
	C	5.7 (8.1)^{A}
Control	Ca	25.6 (1.2)^{A}
	P	20.4 (0.9)^{A}
	C	13.4 (0.5)^{A}

Table 33 Mean atomic % values and standard deviation (SD) of enamel Calcium, Phosphorous, and Carbon after whitening using pH7 6%, 20%, and 40% HP in comparison to a control group. Different superscript letters indicate significant differences between groups.

Surface morphology

Enamel treated with 6% and 20% HP had an overall smooth surface with no visible damage (Figure 61-63). Treatment with 40% HP, on the other hand, lead to the development of craters, deformation of prismatic/inter-prismatic enamel, and the formation of grooves.

6% HP

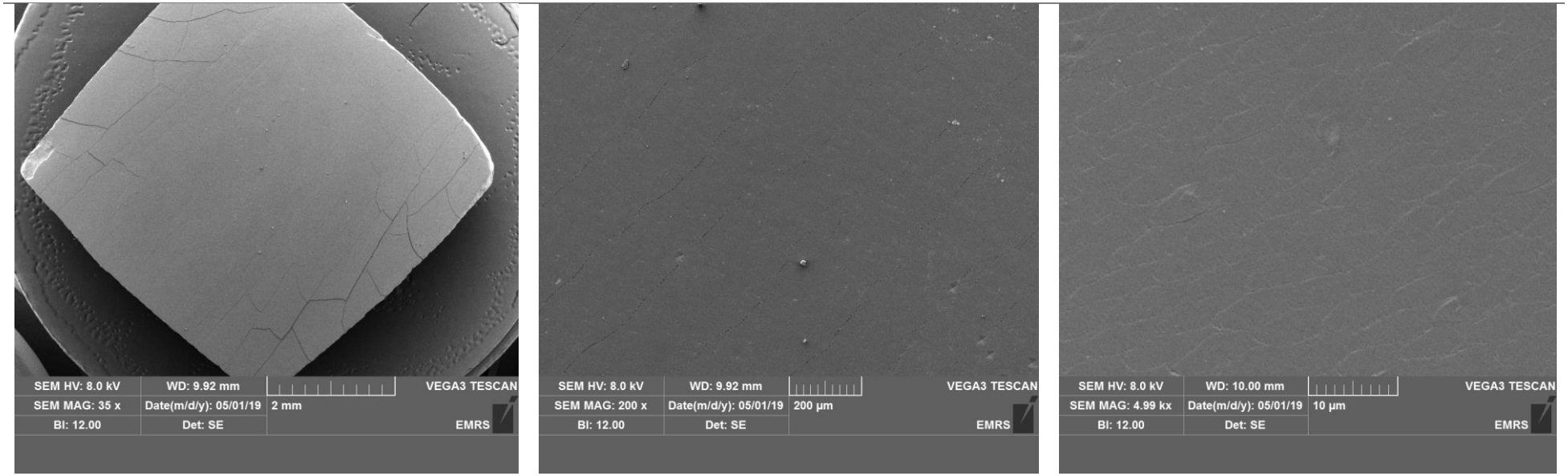


Figure 61 Three SEM images under low, medium, and high magnification of enamel treated with pH7 6% HP. Note the overall smooth surface with visible cracks under low magnification

20% HP

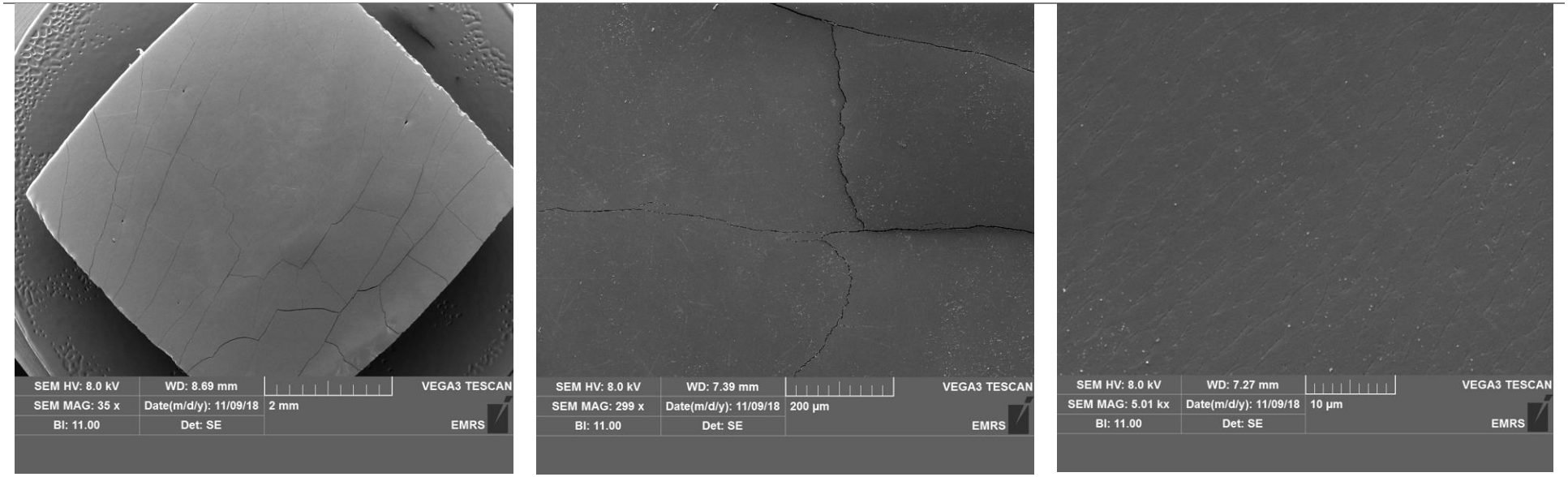


Figure 62 Three SEM images under low, medium, and high magnification of enamel treated with pH7 20% HP. Note the overall smooth surface with visible cracks under low magnification.

40% HP

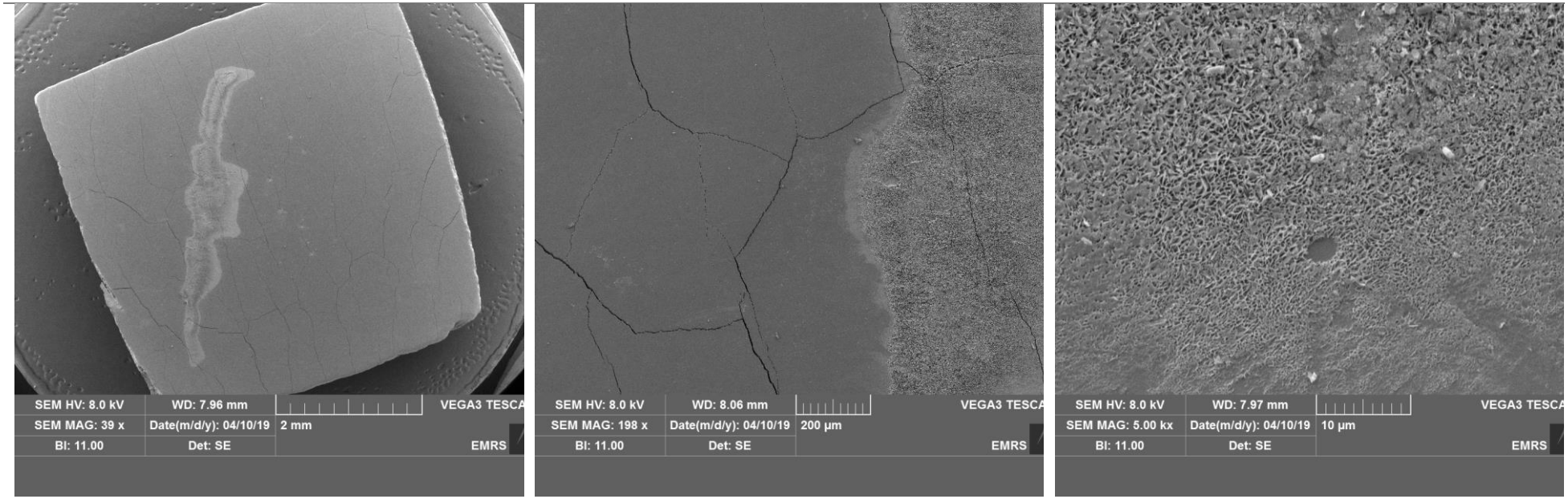


Figure 63 Three SEM images under low, medium, and high magnification of enamel treated with pH7 40% HP. Note the overall smooth surface with visible localised enamel deformation.

5.6.6 Whitening using alkaline hydrogen peroxide

Enamel surface topography

There are no significant differences between enamel treated with 6% HP and control enamel samples (Figure 64). Surface changes occurred in enamel treated with 20% HP; appearing as sharp and narrow peaks separated by deep valleys. Erosive damage was more significant in enamel treated with 40% HP; visible as round and blunt peaks separated by deep valleys.

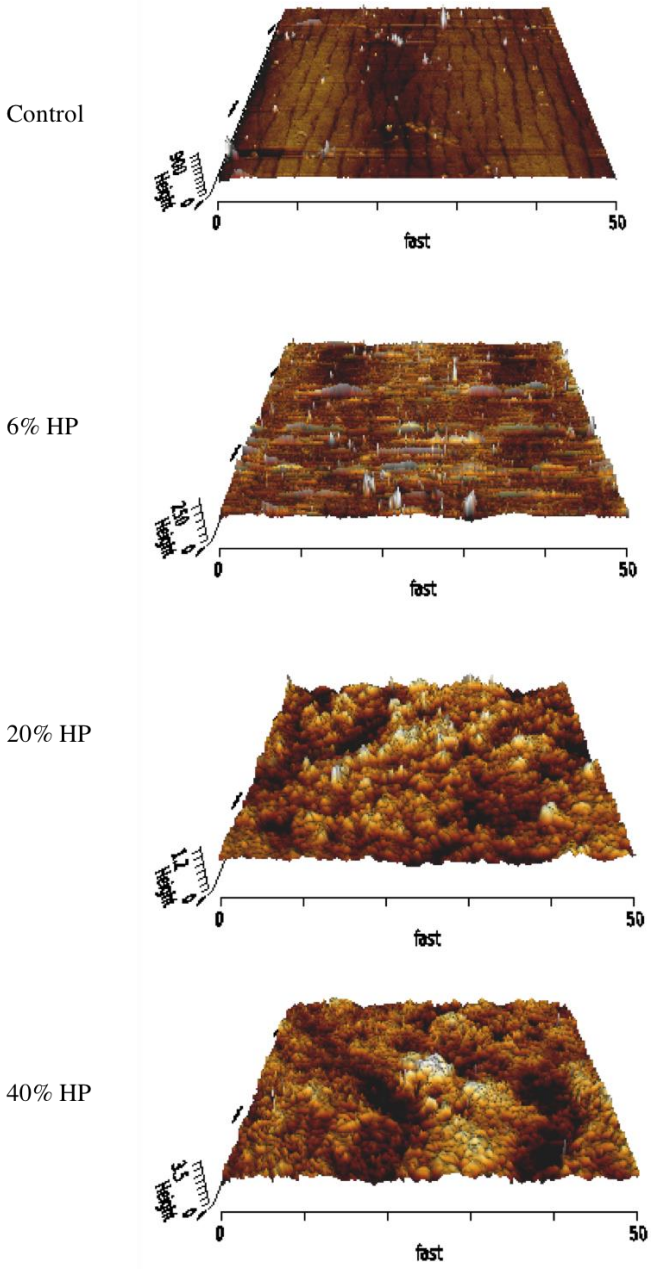


Figure 64 Representative 3D images of a control bovine enamel sample, treated with pH9 6% HP, treated with pH9 20% HP, and treated with pH9 40% HP. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-b) is nm and in images (c-d) is μm.

Enamel roughness (Ra)

Treatment with 6%, 20%, and 40% HP caused a significant increase in enamel Ra (P≤0.05) (Table 34) (Figure 65). The increase in enamel roughness was directly proportional to HP concentration. The 40% HP solution caused a significantly greater increase in enamel Ra in comparison to 20% and 6% HP (P≤0.05). In addition, 20% HP caused a significantly greater increase in enamel Ra in comparison to 6% HP (P≤0.05).

Ra (nm) – pH9		
	Before	After
6%	6.8 (3.0)^{a,A}	10.1 (4.8)^{b,A}
20%	6.8 (4.8)^{a,A}	12.1 (44.0)^{b,B}
40%	5.9 (2.9)^{a,A}	63.6 (217.5)^{b,C}
Control	7.6 (2.8)^{a,A}	6.1 (2.0)^{a,A}

Table 34 Median (IQR) values of enamel Ra before and after treatment using pH9 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

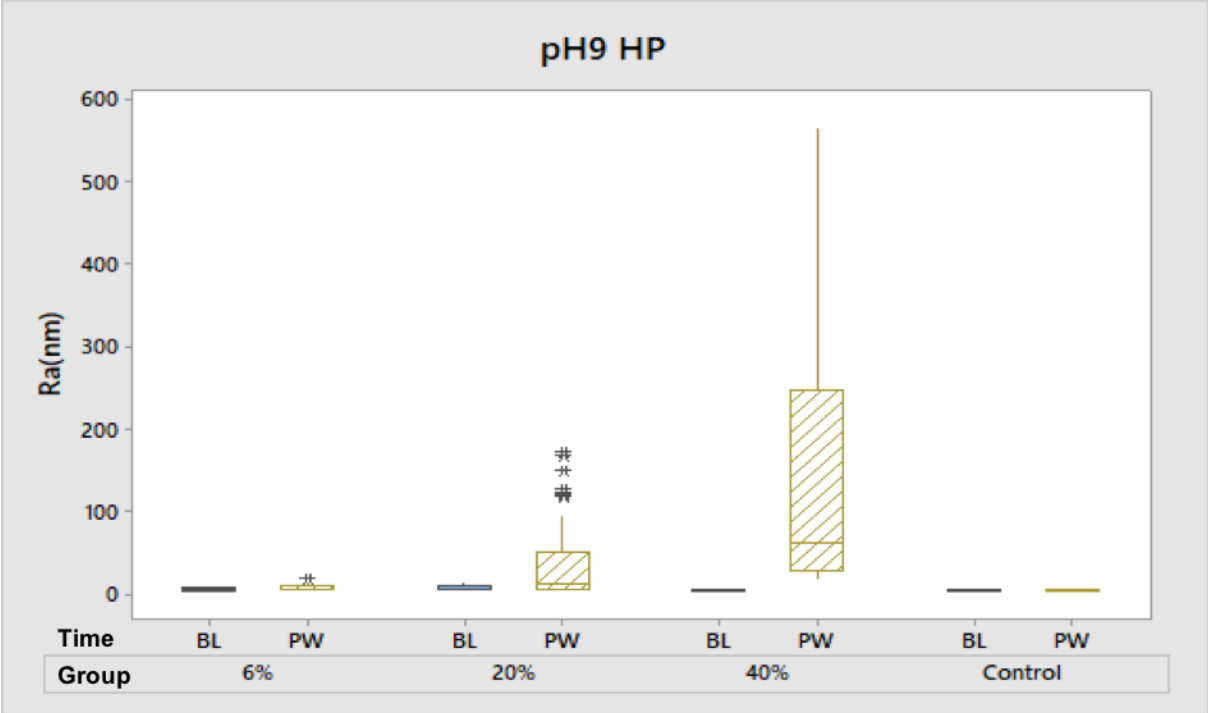


Figure 65 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW) using pH9 6%, 20%, and 40% HP in addition to a control group. Error bars represent the variability of data.

Enamel hardness (HV)

Treatment with 6%, 20%, and 40% HP caused a significant reduction in enamel Vickers hardness (HV) (P≤0.05) (Table 35) (Figure 66). The greatest hardness reduction occurred in enamel treated with 40% HP, which was significantly lower than HV values recorded after treatment with 6% and 20% HP in addition to the control group (P≤0.05).

Hardness (HV) – pH9		
	Before	After
6%	333.7 (28.0)^{a,A}	247.9 (46.3)^{b,A}
20%	327.7 (48.0)^{a,A}	245.6 (81.3)^{b,A}
40%	315.7 (23.4)^{a,A}	118.2 (55.2)^{b,B}
Control	323.1 (97.4)^{a,A}	256.1 (95.4)^{a,A}

Table 35 Median (IQR) values of enamel Vickers hardness before and after treatment using pH9 6%, 20%, 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

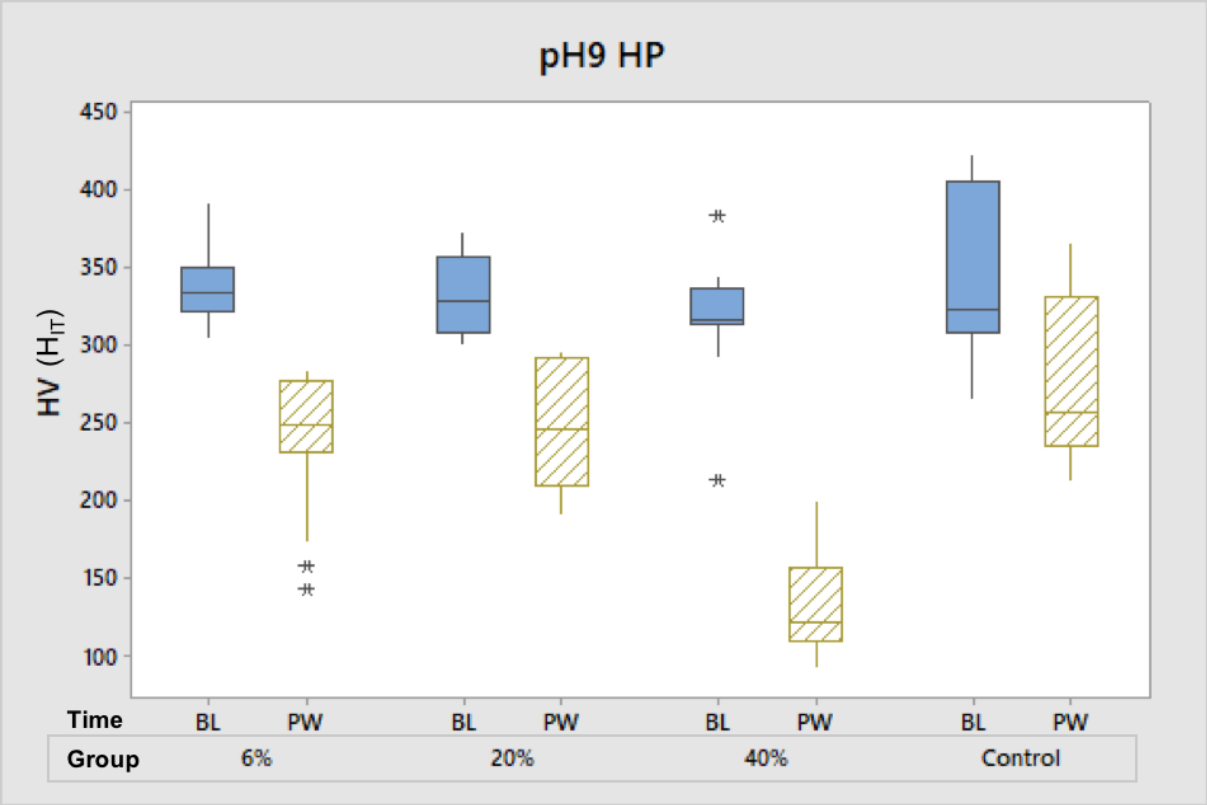


Figure 66 Box and whiskers plot of enamel Vickers hardness at baseline (BL) and post whitening (PW) using pH9 6%, 20%, 40% HP in addition to a control group. Error bars represent the variability of data.

Colour change

Colour change (ΔE) was not significantly different between enamel samples treated with 6%, 20%, and 40% HP (P>0.05). In addition, there were no significant changes in ΔE in the control group (ΔE≤2) (Table 36). A significant increase in lightness (L*) occurred in enamel samples treated with 20% and 40% HP (P≤0.05). Lightness values were significantly greater in enamel treated with 40% HP in comparison to other treatment groups (P≤0.05). A significant decrease in (a*) values towards the green spectrum and (b*) values away from the yellow towards the blue spectrum occurred in enamel whitened using 6%, 20%, and 40% HP (P≤0.05). Changes in (a*) values were not significantly different between whitened enamel samples (P>0.05). The reduction in (b*) values were significantly greater in enamel treated with 6% HP in comparison to enamel treated with 40% HP (P≤0.05).

ΔE			
6%	20%	40%	Control
8.4 (1.2)^{a}	8.3 (1.9)^{a}	9.5 (2.4)^{a}	1.6 (1.1)^{b}
L*			
	Before	After	
6%	82.7 (2.2)^{a,A}	85.3 (1.9)^{a,A}	
20%	81.4 (1.2)^{a,A}	86.4 (1.3)^{b,A}	
40%	82.0 (2.3)^{a,A}	89.2 (0.7)^{b,B}	
Control	80.8 (1.3)^{a,A}	82.2 (0.8)^{a,C}	
a*			
	Before	After	
6%	-0.9 (0.3)^{a,A}	-1.4 (0.1)^{b,A}	
20%	-0.9 (0.3)^{a,A}	-1.3 (0.2)^{b,A}	
40%	-0.7 (0.5)^{a,A}	-1.5 (0.1)^{b,A}	
Control	-0.8 (0.4)^{a,A}	-0.6 (0.5)^{a,B}	
b*			
	Before	After	
6%	8.3 (1.6)^{a,A}	0.5 (0.6)^{b,A}	
20%	8.5 (2.3)^{a,A}	1.9 (1.2)^{b,A,B}	
40%	8.5 (1.6)^{a,A}	2.6 (0.7)^{b,B}	
Control	9.8 (1.0)^{a,A}	11.7 (1.8)^{a,C}	

Table 36 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of enamel before and after treatment using pH9 6%, 20%, and 40% HP in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with 6%, 20%, and 40% HP, atomic % of calcium and carbon were not significantly different than the those recorded in the control group ($P>0.05$) (Table 37). Treatment with 40% HP, however, caused a significant increase in enamel phosphorus in comparison to other treatment groups ($P\leq0.05$).

	Element	Atomic %
6%	Ca	27.7 (1.2)^{A}
	P	20.1 (1.0)^{A}
	C	13.0 (0.1)^{A}
20%	Ca	26.4 (0.3)^{A}
	P	20.0 (0.3)^{A}
	C	13.5 (1.3)^{A}
40%	Ca	25.4 (1.2)^{A}
	P	24.6 (0.7)^{B}
	C	8.7 (2.8)^{A}
Control	Ca	25.6 (1.2)^{A}
	P	20.4 (0.9)^{A}
	C	13.4 (0.5)^{A}

Table 37 Mean atomic % values and standard deviation (SD) of enamel Calcium, Phosphorous, and Carbon after whitening using pH9 6%, 20%, and 40% HP in comparison to a control group. Different superscript letters indicate significant differences between groups.

Surface morphology

Enamel treated with 6% HP showed minor surface deformation and localised deposition of crystals thought to be alkaline salts (Figure 67-69). Treatment with 20% and 40% HP lead to the development of craters, deformation of prismatic/inter-prismatic enamel, and the formation of grooves.

6% HP

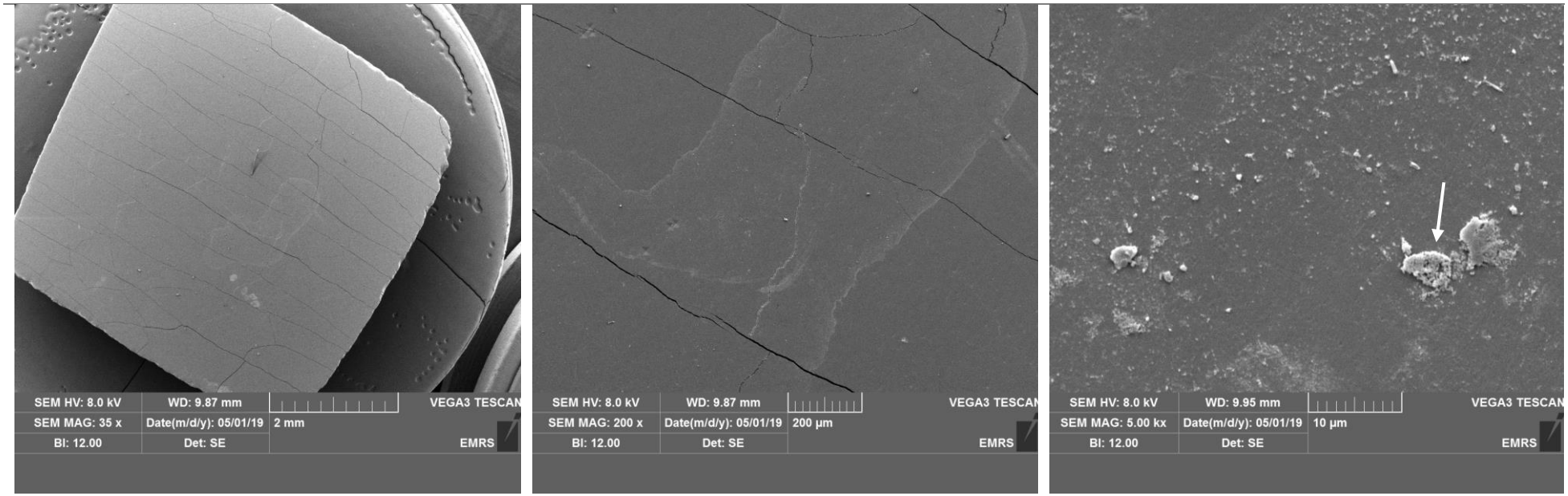


Figure 67 Three SEM images under low, medium, and high magnification of enamel treated with pH9 6% HP. Note the localised deposition of alkaline salts (arrow) along with minor surface deformation under high magnification.

20% HP

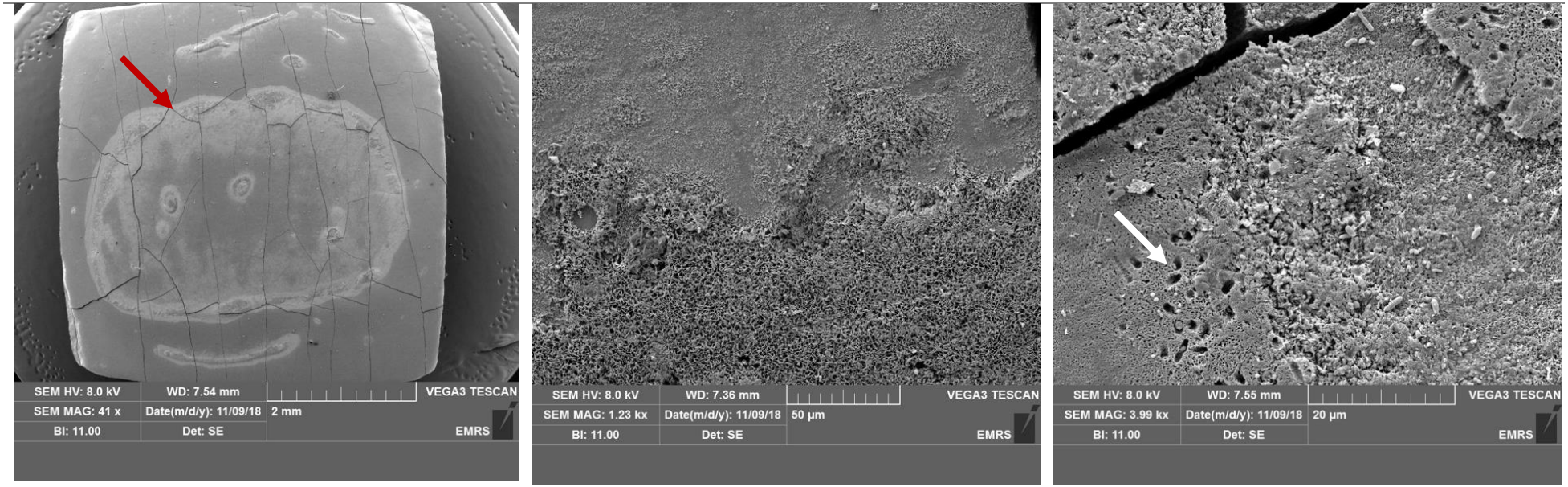


Figure 68 Three SEM images under low, medium, and high magnification of enamel treated with pH9 20% HP. Note the development of craters and the presence of deformed prismatic and inter-prismatic enamel under medium and high magnification. Under high magnification enamel pits are visible (white arrow), and under low magnification demineralisation of enamel can be clearly seen (red arrow).

40% HP

Figure 69 Three SEM images under low, medium, and high magnification of enamel treated with pH9 40% HP. Note enamel deformation by the development of pits, craters (arrow), and grooves under high magnification.

5.7 Discussion

The present study illustrates the impact of HP concentration and pH on bovine enamel. Commercially available HP based whitening products were reported to range from 3% to 40% (Majeed *et al.*, 2015a; Acuña *et al.*, 2019), therefore, three HP concentrations were selected, low; 6%, intermediate; 20%, and high; 40%. Each concentration was divided into three pH values of 5, 7, and 9; representing acidic, neutral, and alkaline whitening agents. Samples were whitened for 2 hours, daily for ten days, which is in line with tray based whitening protocols usually prescribed for 2-4 hours/daily, for 10-14 days (Stokes *et al.*, 1992; Kihn, 2007; Mondelli *et al.*, 2009). Enamel roughness, hardness, and colour measurements were obtained before and after treatment. Qualitative evaluation of enamel microstructure and quantitative assessment of mineral composition were obtained after treatment.

5.7.1 Enamel roughness (Ra)

Arithmetic average roughness (Ra) is the most frequently used parameter to measure enamel roughness after whitening (Fujii *et al.*, 2011). Constituents released by the breakdown of CP or HP following a whitening procedure create porosities, grooves, and cracks in enamel, making it rough and more susceptible to extrinsic staining (Shannon *et al.*, 1993; Pinto *et al.*, 2004; Tredwin *et al.*, 2006; Eva *et al.*, 2013). Current results revealed that enamel Ra is directly proportional to HP concentration, which is in line with previously published studies (Pinto *et al.*, 2004; Moraes *et al.*, 2006; Bistey *et al.*, 2007). Greater concentrations of HP contribute to deeper enamel surface grooves and rougher surface peaks which ultimately lead to greater Ra readings (Hegedüs *et al.*, 1999). Images obtained using AFM helped assess overall surface changes in treated enamel samples, however, the automatic selection of the z-scale by the AFM software made interpretation of subtle differences between groups difficult. Therefore, combining 3-D surface topography with roughness measurements provided a better understanding of enamel surface changes both qualitatively and quantitatively.

According to current results, solution pH greatly impacts enamel Ra at each HP concentration level. Whitening using 6% HP resulted in no significant increase in Ra values at pHs 5 and 7, which is in agreement with previously published research (Ren *et al.*, 2009). On the other hand, results have shown that pH9 HP significantly increased enamel Ra by 49%. This could be attributed to the greater HP:NaOH ratio required for the creation of a pH9 solution (Rodrigues *et al.*, 2005). Mixing NaOH (sodium hydroxide) with HP creates an auto-accelerating reaction which generates oxygen and heat; leading to irreversible damage to enamel microstructure (Rodrigues *et al.*, 2005). The structural damage alkaline pH inflicts on enamel involves the breakdown of organic matter which normally encapsulates enamel prisms and connects prisms

to each other, and this structurally undermines the integrity of the enamel surface and subsurface structures (Taube *et al.*, 2010).

Current results have also shown that the level of surface damage caused by pH9 solutions is directly proportional to the HP concentration. Enamel treated with pH9 20% and 40% HP (Figure 70), showed various degrees of frosty opaque regions on the treated enamel surface, more so in samples treated with 40% HP. Surface deformation observed caused a significant increase in enamel Ra and contributed to the great variability in recorded roughness values; 12.1nm ±44.0 in enamel treated with pH9 20% HP and 63.6nm ±217.5 in enamel treated with pH9 40% HP. According to the literature, alkaline HP based whitening products were reported to have less damaging effects on enamel (Ito and Momoi, 2011; Young *et al.*, 2012; Torres *et al.*, 2014). Following whitening treatments using 3% to 13.5% HP and 6% to 35% CP with pH values ranging from 4.5 to 10.8, a significant increase in enamel Ra was only recorded in samples treated with acidic 13.5%HP (pH6.1) and 35% CP (pH4.9) (Azrak *et al.*, 2010). Differences between these studies and current results could be attributed to the composition of whitening agents selected, materials used to adjust the pH value; such as sodium bicarbonate (Ito and Momoi, 2011) as opposed to NaOH used in this study, in addition to the method of Ra measurement; such as optical profilometry (Azrak *et al.*, 2010) as opposed to AFM. The degree of ionisation of buffering agents such as sodium bicarbonate and NaOH vary, and this might affect the chemical interaction between HP and enamel and its impact on enamel's surface properties (Ito and Momoi, 2011). In addition, roughness readings obtained using AFM, optical and mechanical profilometers, and confocal laser scanning microscopy are not directly comparable due to factors relating to sample surface topography such as isolated particles which impact these instruments differently (Duparré *et al.*, 2002). Consensus on the effectiveness of alkaline HP in producing a greater whitening effect with minimum enamel damage, however, did not exist in the literature. Research revealed that alkaline HP alters enamel morphology through accelerating the oxidation reduction reaction leading to irreversible enamel damage (Araujo *et al.*, 2013; Jurema *et al.*, 2018). The increase in HP:NaOH ratio in alkaline solutions will contribute to an excessive generation of oxygen and heat which is damaging to enamel organic matrix (Rodrigues *et al.*, 2005; Araujo *et al.*, 2013).

The pursuit for an optimal whitening agent pH has lead scientists to formulate a neutral HP. According to the current study, treatment using 6% and 20% HP with a pH value of 7 did not significantly alter enamel roughness. This is in agreement with previously published research, reporting that neutral HP has less damaging effects on whitened enamel roughness; attributed to minor quantities of alkaline salts present in the HP solution evenly adhering to the enamel surface and reducing direct contact between HP and enamel, thus, forming a protective layer

(Sun *et al.*, 2011). Treatment with 30% HP with a pH value of 7 was reported to cause no significant changes to surface topography according to AFM images (Sun *et al.*, 2011); similar to results obtained in this study. According to Çobankara *et al*., enamel treated with neutral (pH7) 10% and 15% CP showed no significant changes in enamel Ra (Çobankara *et al.*, 2004). For this reason, neutral whitening agents are preferred due to their limited adverse effects on enamel microstructure (Sun *et al.*, 2011). At a higher HP concentration of 40%, on the other hand, current results revealed the doubling of enamel Ra, which is possibly caused by the greater quantity of added NaOH; required to adjust the solution pH. In a study where enamel samples were whitened using 40% HP with a pH value of 7, enamel Ra was reported to significantly increase by 38% (Polydorou *et al.*, 2018). Different neutral HP concentrations: 6% and 40% HP and 16% CP were tested and significant differences between recorded enamel Ra after treatment were reported. At a fixed neutral pH, differences in recorded enamel Ra are caused by the whitening agent concentration, which is in accordance to current results; only showing a significant increase in enamel Ra after treatment with neutral 40% HP as opposed to lower concentrations tested.

Similarly, pH5 HP caused no significant Ra changes in enamel at a concentration of 6%, on the other hand, a significant increase in Ra by 25% was recorded in enamel treated with 20% HP. It is interesting, however, that the pH5 solution showed no significant effect on enamel Ra after treatment with 40% HP. Greater concentrations of an acidic HP could have potentially softened the enamel structure, and therefore, contributed to underestimating the level of damage exerted on whitened enamel (Ren *et al.*, 2009; Fujii *et al.*, 2011; Carey, 2014). This can be confirmed by the load-displacement curves illustrated in Figure 71, revealing a greater maximum indentation depth in enamel treated with pH5 40% HP in comparison to other treatment groups. This means that the indenter was able to deeply penetrate the treated enamel, indicating a softer surface more susceptible to elastic and plastic deformation. According to a study conducted by Xu *et al*., whitening enamel using 30% HP with a pH value of 5 resulted in significant erosive deformation to treated enamel samples in comparison to neutral and alkaline HP solutions (Xu *et al.*, 2011). At higher concentrations of whitening agents, there is an increased risk of enamel demineralisation and root resorption upon prolonged exposures to highly acidic products with pH values falling below 5.2 or highly alkaline products with pH above 7 (Driessens *et al.*, 1986; Price *et al.*, 2000).

Figure 70 Shows surface damage visibly noted on a bovine enamel sample treated with pH9 20%HP (left) in comparison to a sample treated with pH9 40%HP which shows a greater degree of surface damage (right).

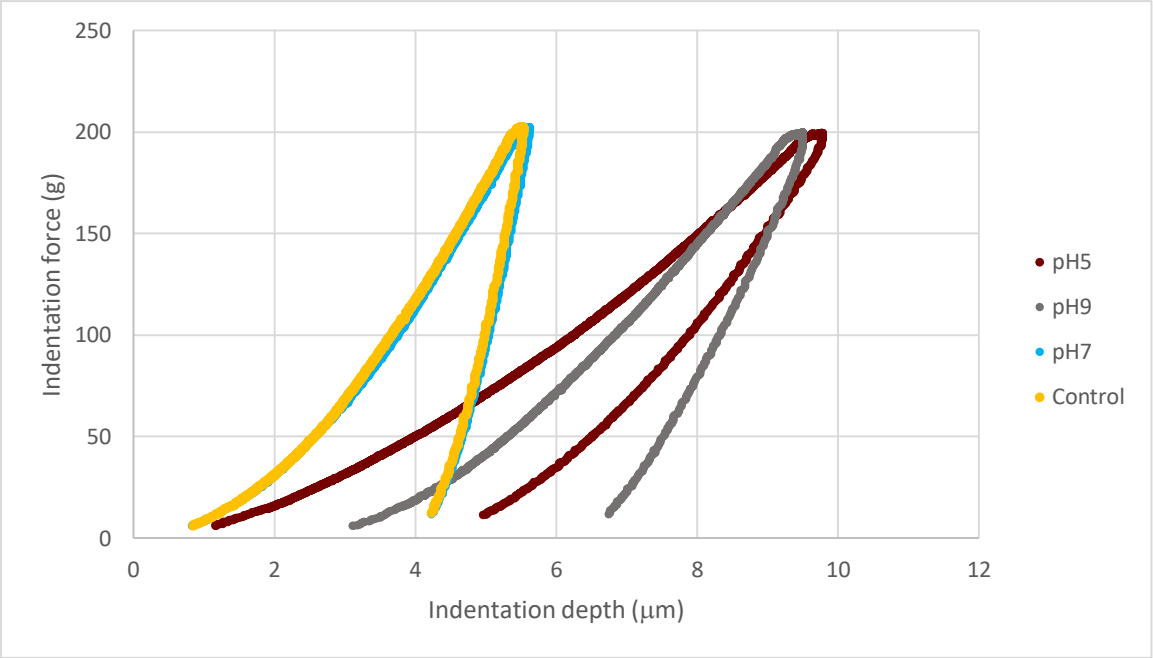


Figure 71 Load displacement curves of bovine enamel treated with pH 5, 7, and 9 40% HP in addition to a control group. Note the similar indentation depth in enamel treated with pH7 HP and the control enamel. In contrast enamel treated with pH5 and 9 HP had a greater recorded indentation depth, more so in the enamel treated with pH9 HP after load removal; indicating a greater degree of plastic deformation.

5.7.2 Enamel Vickers hardness (HV)

Hardness reduction in whitened enamel is caused by the oxidation reduction process initiated by whitening agents such as HP (Markovic *et al.*, 2007; Eva *et al.*, 2013; Jurema *et al.*, 2018). This chemical reaction targets organic and inorganic matter and causes enamel demineralisation, resulting in the development of micro-cracks and porosities which directly affect enamel micro-hardness values. Indeed, a significant reduction in HV values was recorded in bovine enamel after whitening using 6%, 20%, and 40% HP under three pH values of 5, 7, and 9. According to the literature, HP based whitening agents cause significant hardness reductions in treated enamel samples (Attin *et al.*, 2005; George *et al.*, 2015); directly proportional to the concentration of the whitening agent and the duration of the whitening treatment (Al-Salehi *et al.*, 2007; Furlan *et al.*, 2017). The application of whitening agents containing 35% and 15% HP were reported to cause a significant reduction in enamel microhardness (Mondelli *et al.*, 2015). The 35% HP based product caused a significantly greater reduction in enamel hardness in comparison to the 15%HP. According to a study conducted by Sulieman *et al.*, on the other hand, treatment with 35% HP caused no significant changes in enamel hardness values (Sulieman *et al.*, 2004). They claim that damage to enamel/ dentine as a result of whitening is caused by the pH of the whitening agent rather than by its concentration. According to current results, after treatment with 6% and 20% HP, enamel hardness among pH groups was not significantly different, indicating that at a constant HP concentration pH plays no role in minimising the severity of micro-hardness reductions. After treatment with 40% HP, on the other hand, HV values in enamel whitened with pH5 and pH9 HP solutions were significantly lower in comparison to samples whitened using pH7 HP. According to Jurema *et al.*, whitening using 35% HP with pH values of 5, 7, and 8.4, caused a significant reduction in enamel hardness (Jurema *et al.*, 2018). Reductions recorded, however, were not significantly different between pH groups. In contrast, bovine enamel whitened with 35% HP with pH values of 4.3 and 6.6 were reported to exhibit a significant reduction in microhardness values in comparison to baseline (Magalhães *et al.*, 2012). The more acidic product (pH4.3) caused a significantly greater reduction in enamel hardness in comparison to the less acidic product (pH6.6). This was attributed to the pH of the HP solution; being lower than the critical level for enamel (pH5.5) and therefore, leading to enamel demineralising. Both studies (Jurema *et al.*, 2018) and (Magalhães *et al.*, 2012) applied the whitening gel for 30 minutes, however, in the latter study the whitening gel was agitated on the enamel surface after every 1 minute, for a total of 10 minutes. The gel was then rinsed off and the whitening cycle was repeated two more times. According to the literature, the agitation of an acidic gel increases

the rate of enamel demineralisation (Aschheim, 2014), contributing to differences between reported results in the aforementioned studies.

Current results demonstrate the complex interaction between both concentration and pH of HP. Hardness readings have shown that increasing the concentration of an acidic (pH5) or an alkaline (pH9) HP solution will have a greater damaging effect on enamel micro-hardness levels in comparison to a neutral (pH7) HP solution. Treatment with 6%, 20%, and 40% HP solutions with a pH value of 7 caused the same degree of reduction in HV (23-24%). Indeed, enamel hardness values were not significantly affected after treatment with 35% HP with a pH value of 7 (Alexandrino *et al.*, 2014). Increasing the concentration of an alkaline HP solution resulted in the acceleration of free oxygen radical release; causing a greater degree of damage to enamel organic matrix (Araujo *et al.*, 2013). On the other hand, elevating the concentration of an acidic HP solution mostly contributed to a greater level of mineral loss and therefore weaken the whitened enamel as a result (Rodrigues *et al.*, 2005).

5.7.3 Enamel colour

Chromogens are the cause of dental discolouration, and are either present as large organic compounds with double bonds, or as metallic containing compounds, the latter being less likely to be whitened using HP (Carey, 2014). Whitening occurs through the process of chemical degradation of chromogens and whitening products are being continually developed to help improve smile aesthetics with minimal reversible damage to treated enamel surfaces.

Current results have shown a significant increase in enamel ΔE after treatment with 6%, 20%, and 40% HP. The overall ΔE was similar in enamel samples treated with 6% and 20% HP solutions. A significantly greater whitening effect, however, was recorded after treatment with pH5 and pH7 40% HP solutions in comparison to lower concentrations tested. According to previously published studies, there is a directly proportional relationship between whitening agent concentration and the resultant enamel colour (Kawamoto and Tsujimoto, 2004; Fearon, 2007; Borges *et al.*, 2015). However, whitening agent pH is an important factor to consider. The application of 6%, 20%, and 40% HP with a pH value of 9 resulted in similar ΔE values in treated enamel samples. This means that increasing the concentration of an alkaline HP solution from 6% to 40% will have no added benefit in improving enamel colour.

Based on results obtained, changing the pH of 6% HP solutions had no significant effect on the resultant enamel colour after treatment. Similarly, studies examining the effects of HP pH showed no significant differences in ΔE values between enamel samples treated with acidic, neutral, or alkaline HP (Sun *et al.*, 2011; Xu *et al.*, 2011; Sa *et al.*, 2012a; Balladares *et al.*, 2019). The lack of significant differences could be explained by the low HP concentration used in the current study (6%HP), as solution pH appeared to significantly affect enamel ΔE as HP concentration increased to 20% and 40%. At each concentration level pH5 and pH7 HP solutions caused a greater whitening effect in comparison to the pH9 solution. According to the literature, increasing the HP concentration results in elevating its acidity (Weiger *et al.*, 1993; Trentino *et al.*, 2015; Soares *et al.*, 2016a) and in order to adjust the pH of such an acidic solution to create an alkaline HP, greater quantities of NaOH will be required. As previously mentioned, increasing the HP:NaOH ratio will contribute to an excessive generation of oxygen and heat (Rodrigues *et al.*, 2005) which will potentially cause enamel surface damage similar to that noted in Figure 70. This will in turn affect enamel translucency and gloss and as a result enamel colour will be significantly compromised. At 20% and 40% HP concentrations, the whitening effect of pH5 and pH7 solutions were not significantly different. This is in line with previously published research, where enamel samples treated with acidic and neutral 40% HP showed no significant colour differences between treatment groups (Acuña *et al.*, 2019). In

addition, slightly lower concentrations of acidic and neutral HP ranging from 35% to 38% were reported to produce the same level of enamel whiteness (Sa *et al.*, 2012a; Loguercio *et al.*, 2017). This could be explained by the protective salivary pellicle, possibly inhibiting surface erosion; by only allowing the small free radicals to penetrate treated enamel surfaces. It has been reported that acidic agents reduce enamel translucency and increase the opaqueness of the underling dentine layer; producing a frosted glass effect which gives an illusion of a lighter surface (Xu *et al.*, 2011). Therefore, minimising erosive changes in whitened enamel by storing samples in artificial or natural saliva could possibly result in similar ΔE values between enamel treated with acidic and neutral HP.

Enamel lightness values (L*) significantly increased after treatment with 6%, 20%, and 40% HP. Recorded changes were directly proportional to HP concentration, which is in line with previously published research (Kawamoto and Tsujimoto, 2004; Fearon, 2007; Soares *et al.*, 2015). According to an *in-vivo* study, whitening enamel using 6.5% HP and 10% CP caused a significant increase in recorded L* values (Karpinia *et al.*, 2002). This is attributed to oxygen radicals released by HP which oxidise organic chromogens and ultimately reduce tooth discolouration (Carey, 2014). Elevating the concentration of a pH5, 7, and 9 HP solution from 6% to 20% had no added benefit in respect to enamel lightness. The 40% HP, on the other hand, produced significantly greater L* values post treatment in comparison to other concentrations tested. According to a study conducted by Borges *et al*., recorded L* values in enamel samples treated with 35% HP were greater in comparison to enamel treated with 20%, 25% and 30% HP (Borges *et al.*, 2015). Higher concentrations of HP provide a better whitening effect and suggest using such high concentrations when a faster whitening outcome with minor surface changes are desired.

Current results additionally showed similar lightness values in enamel samples treated with pH5, pH7, and pH9 HP solutions at each concentration tested. According to a study conducted by Xu *et al*., treatment using 30% HP with pH values of 7 and 8 resulted in similar changes in enamel lightness, which were significantly greater than that recorded in enamel treated with pH5 HP (Xu *et al.*, 2011). On the other hand, whitening effects of acidic and neutral HP were not significantly different according to an *in-situ* study (Sa *et al.*, 2012a). Differences could be attributed to the composition of whitening agents, whitening methodology, or the storage solution used; stored dry (Xu *et al.*, 2011), as opposed to being stored in natural saliva (Sa *et al.*, 2012a). Enamel samples whitened and stored in artificial saliva, as done in the current study, were reported to exhibit a colour change similar to that recorded in samples stored in natural saliva (Zeczkowski *et al.*, 2015). Both storage solutions were reported to remineralise enamel

after each whitening cycle; possibly affecting the resultant enamel colour and surface properties (Kielbassa *et al.*, 2001).

After treatment with 6%, 20%, and 40% HP, changes along the green-red spectrum (a*), were not significantly different between concentration and pH groups, similar to previously published research (Xu *et al.*, 2011). Majority of whitening studies focus on changes in ΔE values, giving little attention to other colour parameters. Subjective evaluation of colour change following a whitening procedure is strongly associated with changes in (b*) values from yellow to blue than with changes in L* and a* values (Gerlach *et al.*, 2002). Current results revealed that favourable changes along the yellow-blue spectrum (b*) post whitening, towards the blue end, did not decrease beyond -0.1, which was established by 6% HP, irrespective of pH values tested. Raising the concentration of HP to 20% or 40% did not significantly improve what has been established by the 6% HP. In this case, increasing the HP concentration and varying HP pH did not significantly impact the resultant a* and b* readings. It would be interesting, however, to test if HP concentrations lower than 6% could achieve the same degree of colour change along these two parameters.

5.7.4 Enamel mineral composition

Mature enamel is approximately 96% inorganic matter, mostly hydroxyapatite crystals which mainly consist of calcium (Ca) and phosphorus (P), in addition to 1-2% organic matter and 2-3% water (Gutiérrez-Salazar and Reyes-Gasga, 2003; Zaichick and Zaichick, 2014). The organic matter mainly contains carbon (C) which is also present as carbonate radicals usually associated with enamel inorganic matter (Watson *et al.*, 1967). Biological hydroxyapatite is often described as calcium-deficient hydroxyapatite due to impurities normally present, which is caused by the constant replacement of ions by other ions with the same sign and a different charge in an attempt to maintain neutrality (Lu *et al.*, 2000; Taube *et al.*, 2010). In comparison to pure hydroxyapatite, carbonated hydroxyapatite crystals, for example, involve the replacement of Ca-PO_4 strong bond with a weak Ca-CO_3 bond (Taube *et al.*, 2010; Elliott, 2013). This contributes to the reduction of the apatite crystal size and increase the apatite crystal strain. Carbonated hydroxyapatite, along with calcium-deficient hydroxyapatite, is therefore more susceptible to erosive attacks than pure hydroxyapatite.

In order to estimate the level of damage HP concentration and pH inflict on enamel mineral composition, the atomic percentages of Ca, P, and C were quantified by EDX. The atomic percent indicates the percentage of one kind of atom in relation to the total number of atoms present, and has been used to quantify and compare enamel elemental components in demineralisation, remineralisation, and whitening studies (Ceballos-Jiménez *et al.*, 2018; Ahmed, 2019; Scholz *et al.*, 2019). In this study, carbon was measured to assess the level of damage on enamel organic matter which has been reported to be highly susceptible to alkaline pH and high HP concentrations (Taube *et al.*, 2010). The inorgainc matter, on the other hand, is highly susceptible to high HP concentrations and low pH values and is assessed by measuring Ca and P (Driessens *et al.*, 1986; Price *et al.*, 2000).

All HP concentrations and pH values tested caused no significant changes in enamel Ca and C. The atomic % of P, on the other hand, significantly increased in enamel exposed to 40% HP, irrespective of its pH value, in comparison to other concentration groups. The absence of significant changes in Ca, C, and P in enamel treated with 6% and 20% HP with pHs 5, 7, and 9 in this study and in previously published studies (Çakır *et al.*, 2012; Coceska *et al.*, 2016; Ahmed, 2019), indicate that enamel surface mineralisation is not significantly affected by whitening treatments using HP concentrations up to 20%. In addition, the remineralisation effect of the artificial saliva used as a storage medium could have contributed to the absence of any significant changes in enamel minerals (Amaechi and Higham, 2001b; Amaechi and Higham, 2001a; Wang *et al.*, 2011). However, as it appears from current results, treatment with 40% HP statistically increased the atomic % of enamel P. According to Wang *et al.*, EDX

quantification of elemental composition should be interpreted with caution, and must be viewed in terms of relative as opposed to absolute amounts (Wang *et al.*, 2019). Furthermore, studies have shown that the greatest disadvantage of EDX quantitative elemental analysis, is its low energy resolution which could potentially lead to spectrum overlapping and misidentification of elements, therefore, caution and attention are required during analysis (Harada and Ikuhara, 2013).

On the other hand, whitening using 16% CP and 38% HP (Ahmed, 2019), 10% and 38% HP (Pinto *et al.*, 2017), and 10%, 20%, and 35% CP (Cakir *et al.*, 2011), were reported to significantly decrease inorganic and organic elements in treated enamel surfaces. The test substrate must be considered, however, when interpreting published results as human enamel samples were used in the aforementioned studies, and although bovine enamel is the most preferred alternative to human enamel due to their histochemical similarities and similarities in behaviour and response to whitening procedures (Nakamichi *et al.*, 1983; Yassen *et al.*, 2011; Soares *et al.*, 2016a), bovine enamel has greater proportions of inter-prismatic enamel than human enamel which makes it more resistant to acid attacks (Xiao *et al.*, 2009).

5.7.5 Enamel morphology

Dental whitening is intended to improve smile aesthetics without damaging the enamel microstructure. According to the literature, changes in enamel microstructure after whitening depend on the concentration and pH of the whitening agent (McCracken and Haywood, 1996; Taube *et al.*, 2010; Coceska *et al.*, 2016). Indeed, significant damage in enamel mostly occurred after treatment with acidic (pH5) and alkaline (pH9) HP and was directly proportional to HP concentration. It has been reported that highly acidic products with pH values falling below 5.2 or highly basic products with a pH above 7 are more likely to damage and demineralise treated enamel surfaces (Driessens *et al.*, 1986; Price *et al.*, 2000). Current results showed that enamel treated with pH5 HP at 20% and 40% concentrations caused an erosive damage visible as pitting across treated surfaces. Treatment with 25% HP with an acidic pH of 3.2 caused significant surface alterations and demineralisation in treated enamel samples (Eva *et al.*, 2013). Acidic agents were reported to dissolve inorganic matter, causing surface erosions and porosities. Therefore, neutral whitening agents are recommended to minimise adverse effects on enamel microstructure (Pimenta-Dutra *et al.*, 2017). The application of 6% and 20% HP solutions with a pH value of 7 caused no significant alterations to enamel microstructure according to current results. This is in line with previously published research revealing no significant changes in enamel microstructure after whitening using neutral 6% and 25% HP (Lilaj *et al.*, 2019). This could be attributed to minor quantities of alkaline salts present in the HP gel, evenly adhering to the enamel surface and reducing direct contact between HP and enamel, thus, forming a protective layer (Sun *et al.*, 2011).

After treatment with pH9 6%, 20%, and 40% HP, on the other hand, significant enamel damage, directly proportion to HP concentration, was noted. Surface alterations were initially localised showing minor surface deformation, and as the HP concentration increased the area of damage expanded; becoming more porous, deformed, and etched. This could be explained by the greater HP:NaOH ratio required for the creation of a pH9 solution. Mixing NaOH (sodium hydroxide) with HP creates an auto-accelerating reaction which generates oxygen and heat; leading to irreversible damage to enamel microstructure (Rodrigues *et al.*, 2005). According to a study conducted by Taube *et al.*, enamel exposed to alkaline agents with pH values of 14 and 15 exhibited significant surface deformation and loss in the prismatic and interprismatic structures, similar to current results (Taube *et al.*, 2010). This was explained by the dissolution of enamel organic matter and the subsequent loss of the protein rich matrix surrounding enamel prisms. Losing the interprismatic structure will in turn cause the loss of intact prismatic apatite.

Current results have additionally shown that morphological damage in treated enamel samples was directly proportional to HP concentration. Treatment with pH5 and pH7 6% HP caused no

surface alterations to enamel microstructure. This is in accordance to previously published studies where whitening using 6% and 6.5% HP showed no deleterious effects on treated enamel surfaces (White *et al.*, 2003; Duschner *et al.*, 2006). The greatest microstructural damage in the current study was noted in enamel treated with 40% HP. Surface alterations increased in severity as pH values increased. Whitening using 35% HP was reported to cause significant surface damage in treated enamel samples. This was attributed to the large quantity of hydrogen ions present in such high HP concentrations which bind to Ca and P ions; resulting in enamel mineral loss and subsequent surface erosion and structural deformation (Pimenta-Dutra *et al.*, 2017).

5.8 Conclusion

Enamel Ra was significantly affected by HP pH and concentration. Treatment with 6% HP with pH values of 5 and 7 resulted in no significant changes in enamel Ra, however, treatment with a 6% HP alkaline solution (pH9) significantly increase enamel roughness by 49%. Treatment with alkaline 6%, 20%, and 40% HP caused the greatest enamel Ra increase in comparison to acidic and neutral HP solutions. In addition, alkaline HP caused significant morphological and topographical damage to treated enamel samples according to SEM and AFM respectively; being directly proportional to the HP concentration.

Solution pH had no significant effect on enamel micro-hardness (HV) after whitening using 6% and 20% HP. In addition, reductions in enamel micro-hardness (HV) were directly proportional to the concentration of acidic (pH5) and alkaline (pH9) HP solutions. Treatment with 6%, 20%, and 40% HP solutions with a pH value of 7, on the other hand, caused the same degree of reduction in enamel HV (23-24%). Although whitening treatments had a significant effect on enamel hardness, there were no significant changes in enamel mineral composition after whitening.

Acidic HP (pH5) caused the greatest overall colour change (ΔE), and was noted to increase as HP concentration increased. The least colour change was observed after treatment with pH9 solutions in comparison to other pH values tested, showing similar whitening results at pH9 40% HP to enamel treated with pH5 6% HP ($\Delta E = 9.5$).

Surface topography and qualitative assessment of enamel microstructure revealed that pH7 HP caused the least enamel deformation at 6% and 20% concentrations, however, damage was noted after treatment with 40% HP which also had a great detrimental effect at pH5 and pH9 values.

Chapter 6. Phase II: The effects of remineralising agents on whitened enamel

6.1 Introduction

Dental whitening impacts enamel mineral content, hardness, roughness, and colour (Çakır *et al.*, 2012; Coceska *et al.*, 2016; Ahmed, 2019). According to conclusions drawn from the previous chapter, the whitening outcome depends on the concentration and pH of the whitening agent. Whitening using pH5, pH7, and pH9 6% HP resulted in different outcomes on both positive and negative ends of the expected outcome spectrum. Therefore, a novel approach was developed in this study to minimise the harmful side effects witnessed in Phase I by remineralising enamel samples whitened using 6% HP with pH values of 5, 7, and 9. Enamel was remineralised after each whitening cycle for 5 minutes using either an experimental 15.5% nanohydroxyapatite (nHa) or a commercially available 10% CPP-ACP paste. The CPP-ACP paste used is derived from milk protein 'casein' reported to have a high affinity as it chemically bonds to hydroxyapatite present in enamel, maintaining the saturation of calcium and phosphate ions, thus, hindering the demineralisation process caused by bacterial or erosive attacks (Somasundaram et al., 2013).

6.2 Study aims

1- Study the effects of mineralising agents on enamel whitened using pH5, pH7, and pH9 6% HP in relation to enamel colour, roughness, hardness, mineral composition, and enamel microstructure.

2- Compare between the effects of enamel remineralisation using CPP-ACP and nHa in relation to enamel colour, roughness, hardness, mineral composition, and enamel microstructure.

6.3 Materials and methods

Bovine enamel samples (Intertek© group plc., Cheshire, UK) (n=144) were divided into 4 groups (n=36): whitened with pH5 6wt.% HP (Sigma-Aldrich, Inc., Irvine, UK), whitened with pH7 6wt.% HP, whitened with pH9 6wt.% HP, and finally the control group treated with Dulbecco's phosphate buffered saline (PBS) (Gibco©, Paisley, UK) (pH 7.4). HP concentrations and pH values were obtained as previously described in sections 4.2.1 and 4.2.2 and the whitening treatment was carried out as described in section 5.3. After each whitening cycle, enamel samples from each treatment group (n=36) were rinsed with PBS (Gibco©, Paisley, UK) for 10 seconds, air dried for 10 seconds, then remineralised by either applying 10% CPP-ACP (GC tooth mousse, Recaldent™, Henry Schein Laboratory, Gillingham, UK) (n=12) (Figure 72) or 15.5% nHA (NanoXIM CarePaste™, Fluidinova, Moreira da Maia, Portugal) (n=12) (Table 38), or stored back in artificial saliva at 37°C as a control (Hot box oven with fan, Gallenkamp©, Loughborough, UK) (n=12). The mineralising agents were applied using a micro-brush, left for five minutes, then samples were thoroughly rinsed using PBS (Gibco©, Paisley, UK) for 10 seconds, air dried for 10 seconds, then stored back in artificial saliva at 37°C (Hot box oven with fan, Gallenkamp©, Loughborough, UK). Enamel remineralisation was carried out after each whitening cycle, daily, for 10 days. Measurements of enamel roughness, hardness, and colour were investigated before and after treatment as described in section 5.3. In addition, qualitative evaluation of enamel microstructure and quantitative assessment of mineral composition were obtained after treatment.

Nanohydroxyapatite		
		wt%
Components	nHA	15.5±0.5
	KCl	4.5±0.5
	H_2O	80.0±1.0
Particle size	<50 nm	-

Table 38 The composition of the nanohydroxyapatite agent used in this study (NanoXIM CarePaste™, Fluidinova, Moreira da Maia, Portugal).

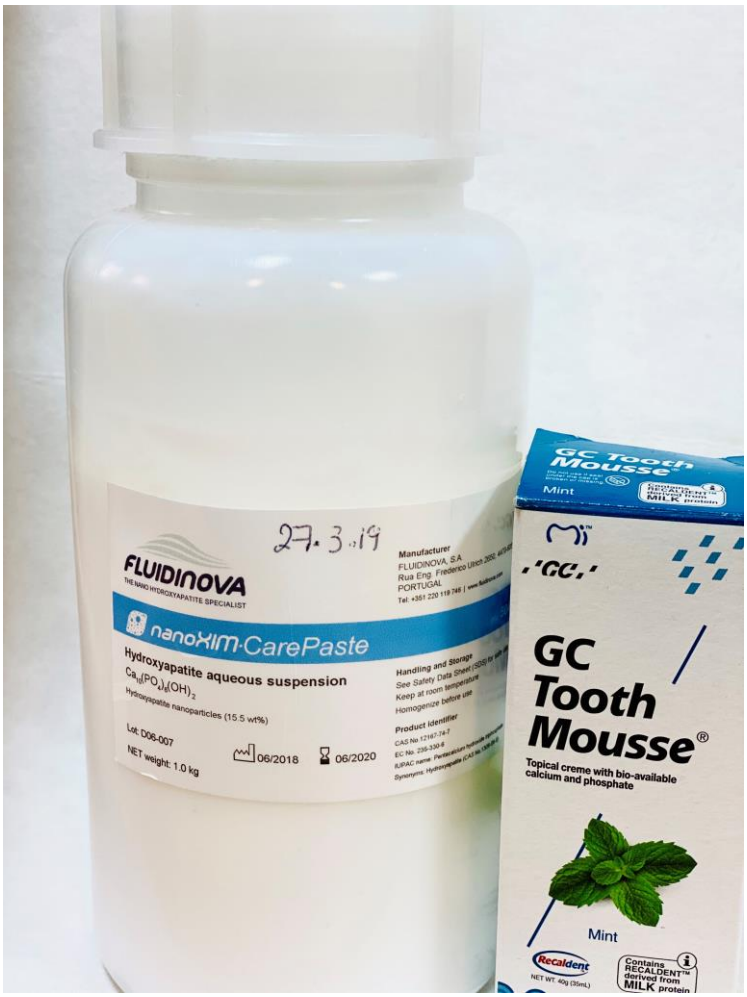


Figure 72 Nano-hydroxyapatite and CPP-ACP pastes used as remineralising agents in the current study.

6.4 Study Design

The programme of work is shown in Figure 73.

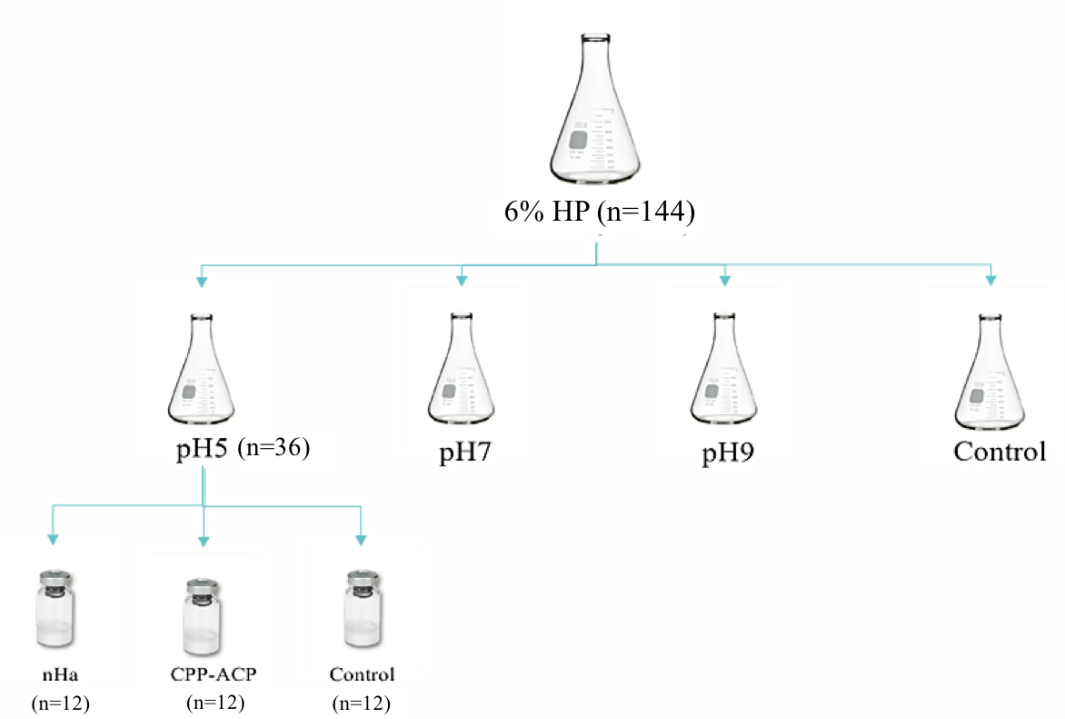


Figure 73 A schematic illustration of the study design. Enamel samples (n=144) were whitened using 6% HP with pH values of 5 (n=36), 7 (n=36), and 9 (n=36) in addition to a control group treated with PBS (n=36). Each treatment group was divided into 3 subgroups where samples were remineralised using nHA (n=12), CPP-ACP(n=12), or kept as a control (n=12).

6.5 Statistical Analysis

Statistical analysis was performed using Sigma Plot© for Windows version 13.0 build 13.0.0.83 (Systat Software 2014®). Statistical analysis of data was performed using Kruskal-Wallis One Way Analysis of Variance on Ranks for non-parametric data, while normally distributed data were analysed using One Way Analysis of Variance. Normality tests were performed using Shapiro-Wilk, and equal variance was tested using Brown-Forsythe. Pairwise multiple comparisons were done using Tukey test with an overall significance of 0.05.

6.6 Results

6.6.1 Whitening using 6% Hydrogen Peroxide at pH5

Enamel surface topography

Enamel remineralised using CPP-ACP showed an overall smooth and un-affected surface, similar to the control enamel, with minor debris present as white spikes (Figure 74). Enamel treated with nHA, on the other hand, revealed significant surface irregularities visible as sharp peaks and deep valleys randomly distributed across the treated surface.

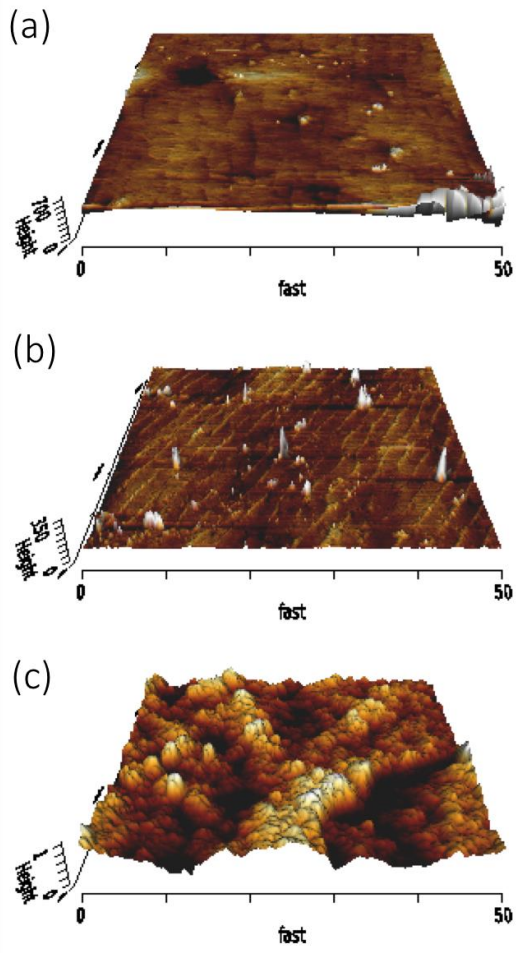


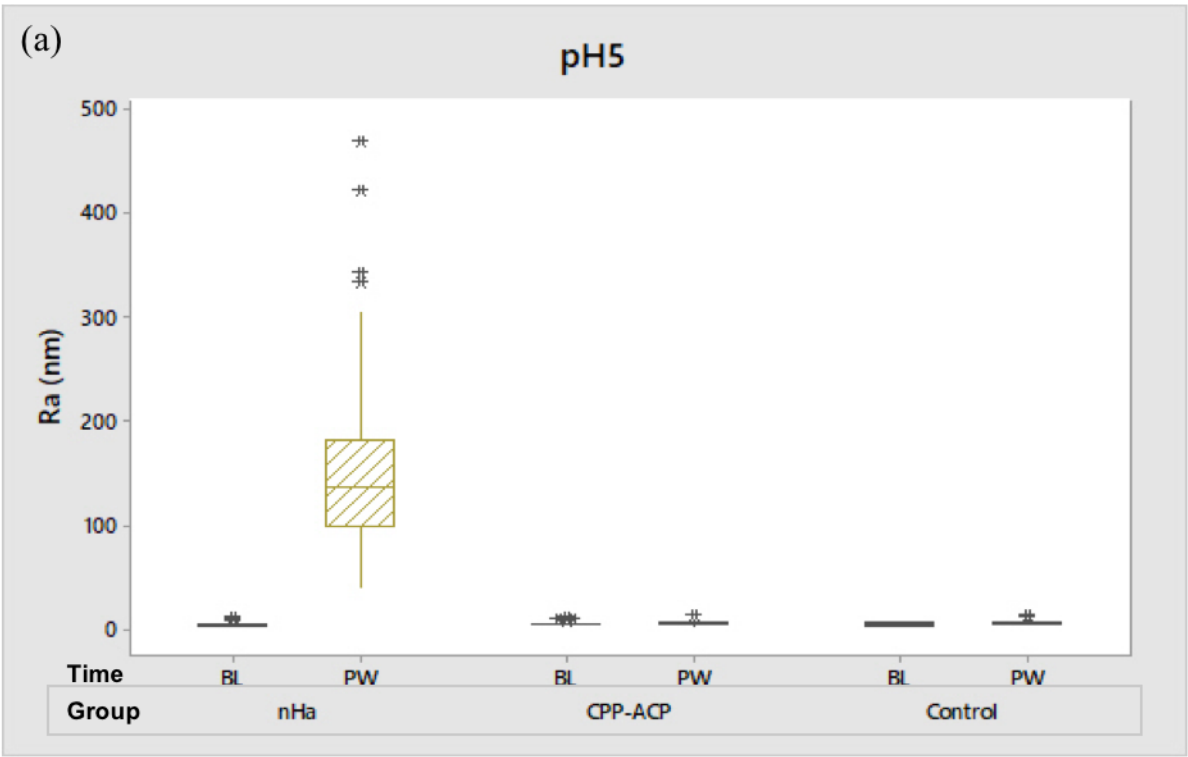
Figure 74 Representative 3D AFM images of whitened bovine enamel samples using 6% HP with a pH value of 5. Samples were divided into three groups; control (a), remineralised with CPP-ACP (b), and remineralised with nHA (c). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-b) is nm and in image (c) is μm.

Enamel roughness (Ra)

After remineralisation with CPP-ACP and nHA a significant increase in enamel Ra was recorded (P≤0.05) (Table 39) (Figure 75). In addition, control enamel samples showed a significant increase in roughness after treatment (P≤0.05). Enamel remineralised using nHA had a significantly rougher surface in comparison to the control group and samples remineralised with CPP-ACP (P≤0.05). Enamel roughness was not significantly different between samples treated with CPP-ACP and control enamel samples (P>0.05).

	Ra (nm)	
Treatment	Before	After
Control	5.2 (2.3)^{a,A}	6.0 (2.8)^{b,A}
CPP	5.4 (1.7)^{a,A}	6.0 (3.1)^{b,A}
nHA	5.5 (1.8)^{a,A}	136.2 (83.0)^{b,B}

Table 39 Median (IQR) Ra values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.



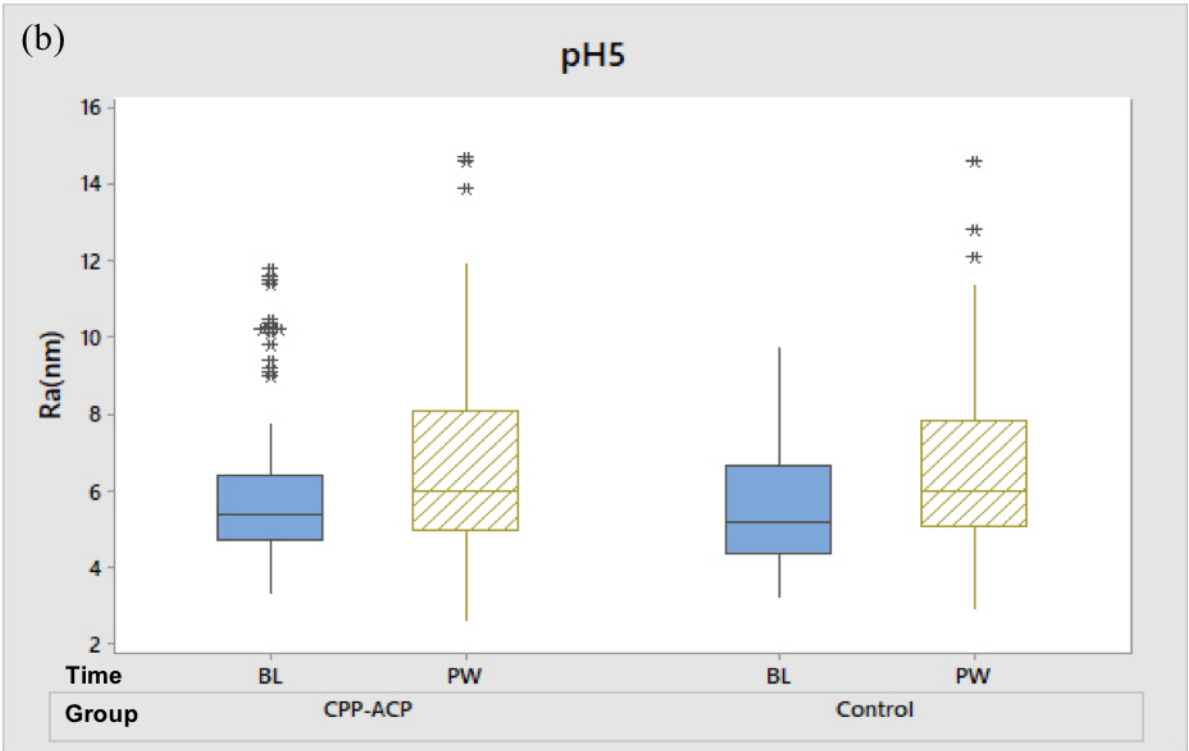


Figure 75 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group (a). Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using CPP-ACP in addition to a control group (b) Error bars represent the variability of data.

Enamel hardness (HV)

After remineralisation with CPP-ACP, a significant decrease in enamel Vickers hardness was recorded (P≤0.05). However, enamel hardness was not significantly different between samples treated with CPP-ACP, nHA , and control enamel samples (P>0.05) (Table 40) (Figure 76).

	HV (H_{IT})	
Treatment	Before	After
Control	324.2 (34.2)^{a,A}	321.5 (34.9)^{a,A}
CPP	330.8 (38.7)^{a,A}	301.1 (56.1)^{b,A}
nHA	331.9 (36.2)^{a,A}	299.7 (113.7)^{a,A}

Table 40 Median (IQR) HV values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

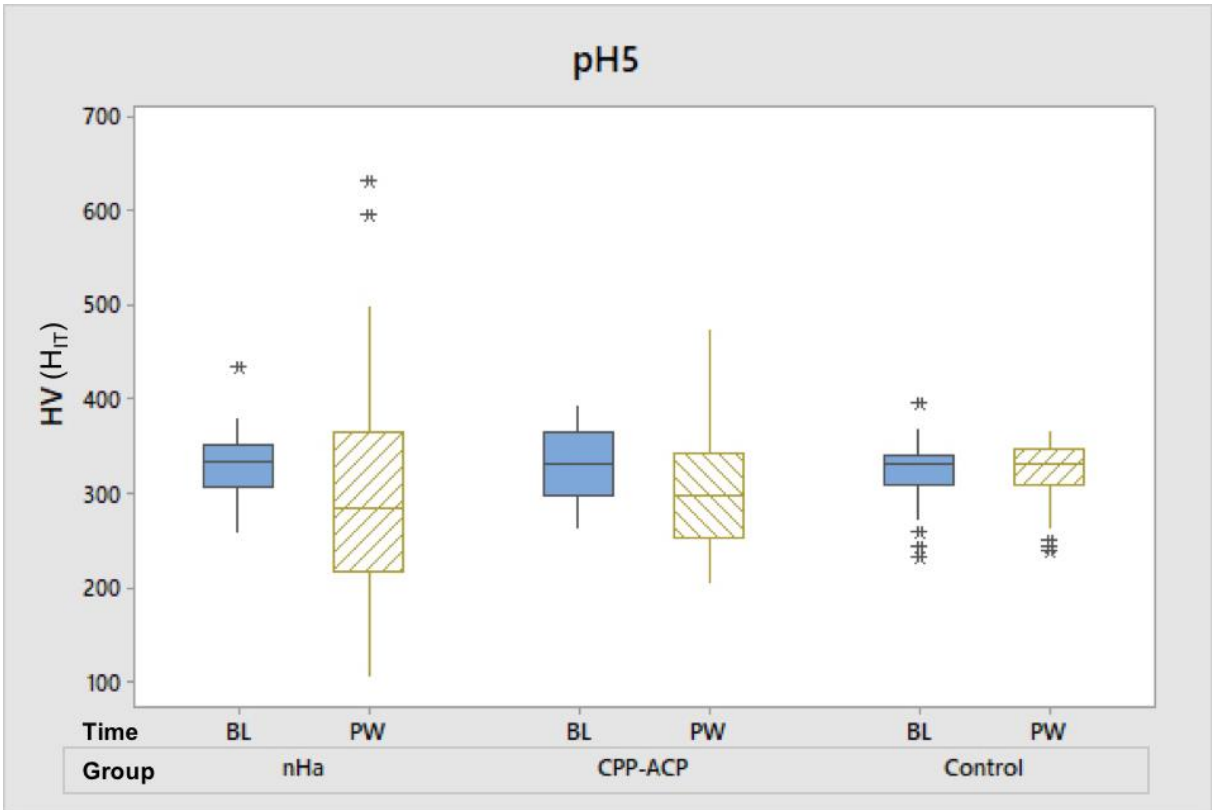


Figure 76 Box and whiskers plot of enamel HV at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group. Error bars represent the variability of data.

Colour change

The greatest change in colour (ΔE) occurred in enamel remineralised with CPP-ACP in comparison to samples treated with nHA and control enamel samples (P≤0.05). A significant increase in enamel lightness (L*), decrease in a* values away from the red and towards the green spectrum, and a significant decrease in b* values away from the yellow towards the blue spectrum were recorded in enamel treated with CPP-ACP, nHA, in addition to the control group (P≤0.05). Enamel lightness (L*) was significantly greater in samples remineralised with CPP-ACP in comparison to control enamel samples (P≤0.05). In addition, reductions in a* values away from the red and towards the green spectrum, and in b* values away from the yellow towards the blue spectrum were significantly different between control enamel samples and enamel remineralised with nHA (P≤0.05) (Table 41).

ΔE		
CPP-ACP	nHa	Control
12.0 (2.2)^{a}	9.5 (2.3)^{b}	9.2 (1.2)^{b}
L* value		
	Before	After
CPP-ACP	81.3 (1.7)^{a,A}	90.8 (1.8)^{b,A}
nHA	81.5 (2.1)^{a,A}	88.2 (1.1)^{b,A,B}
Control	81.4 (1.2)^{a,A}	87.5 (1.0)^{b,B}
a* value		
	Before	After
CPP-ACP	-0.3 (0.5)^{a,A}	-1.2 (0.2)^{b,A,B}
nHA	-0.1 (0.8)^{a,A}	-1.2 (0.1)^{b,A}
Control	-0.5 (0.4)^{a,A}	-1.3 (0.1)^{b,B}
b* value		
	Before	After
CPP-ACP	9.1 (2.3)^{a,A}	1.9 (0.7)^{b,A,B}
nHA	9.1 (2.1)^{a,A}	3.0 (1.05)^{b,A}
Control	8.5 (1.3)^{a,A}	1.8 (1.2)^{b,B}

Table 41 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with CPP-ACP and nHA atomic % of enamel phosphorus and carbon were not significantly different than the those recorded in the control group ($P>0.05$). Enamel treated with nHA had a significantly lower atomic % of calcium than samples treated with CPP-ACP and control enamel (Table 42).

	Element	Atomic %
nHA	Ca	24.0(0.5)[a]
	P	19.8(1.2)[a]
	C	12.8(2.9)[a]
CPP-ACP	Ca	26.4(0.7)[b]
	P	21.3(2.9)[a]
	C	14.2(1.1)[a]
Control	Ca	26.2(0.1)[b]
	P	18.6(1.3)[a]
	C	14.2(5.5)[a]

Table 42 Mean atomic % values and standard deviation (SD) of calcium, phosphorous, and carbon in bovine enamel samples after whitening/remineralisation using CPP-ACP and nHA, in comparison to a control group. Different superscript letters indicate significant differences between groups.

Surface morphology

Enamel remineralised using nHA exhibited a rough irregular surface; caused by the accumulation of nHA crystals. Enamel treated with CPP-ACP and control enamel samples, on the other hand, showed an overall smooth surface with no visible damage (Figure 77-79).

nHA

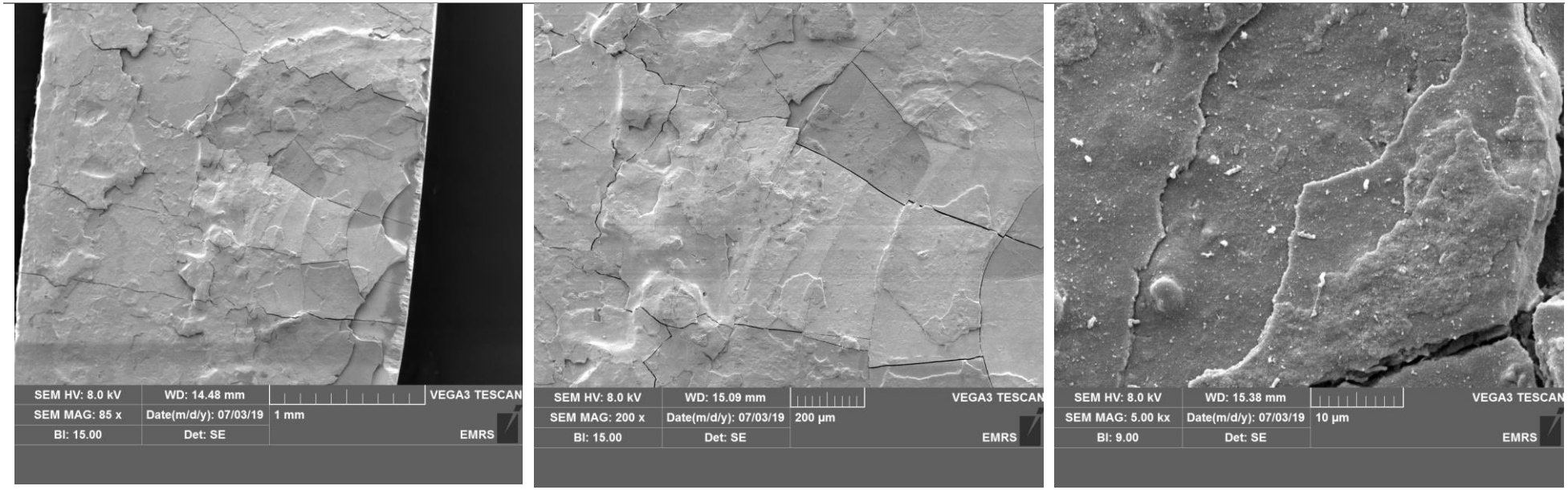


Figure 77 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP and remineralised using 15.5% nHA. Note the rough irregular layer formed by the accumulation of nHA crystals.

CPP-ACP

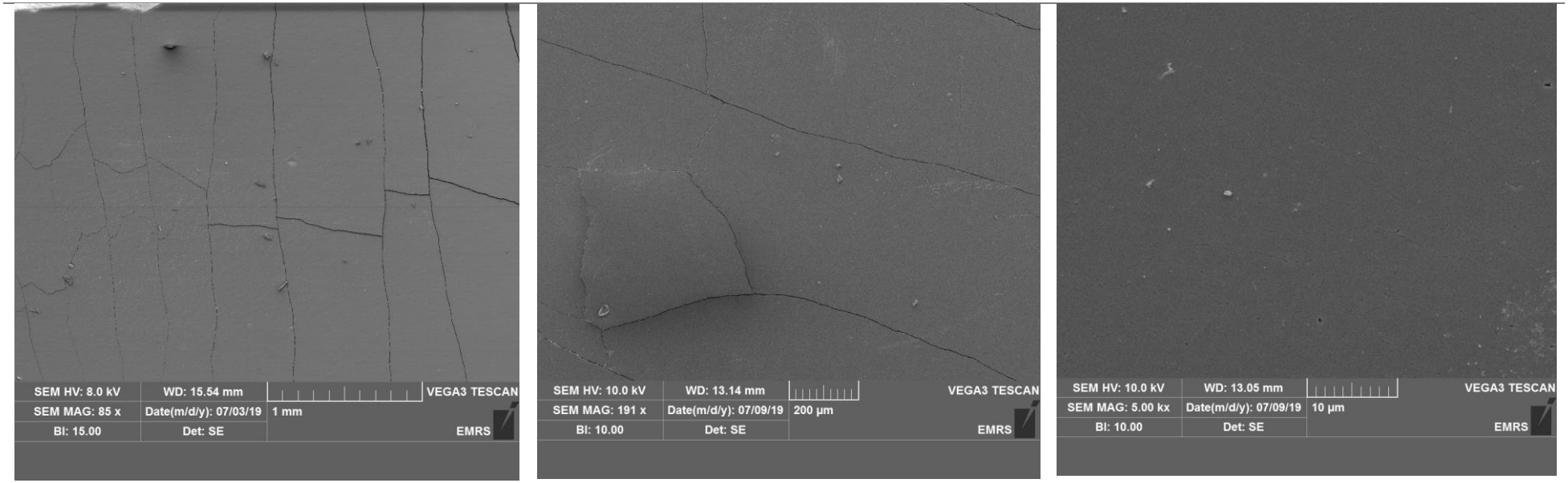


Figure 78 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP and remineralised using CPP-ACP. No significant changes were noted in treated enamel samples.

Control

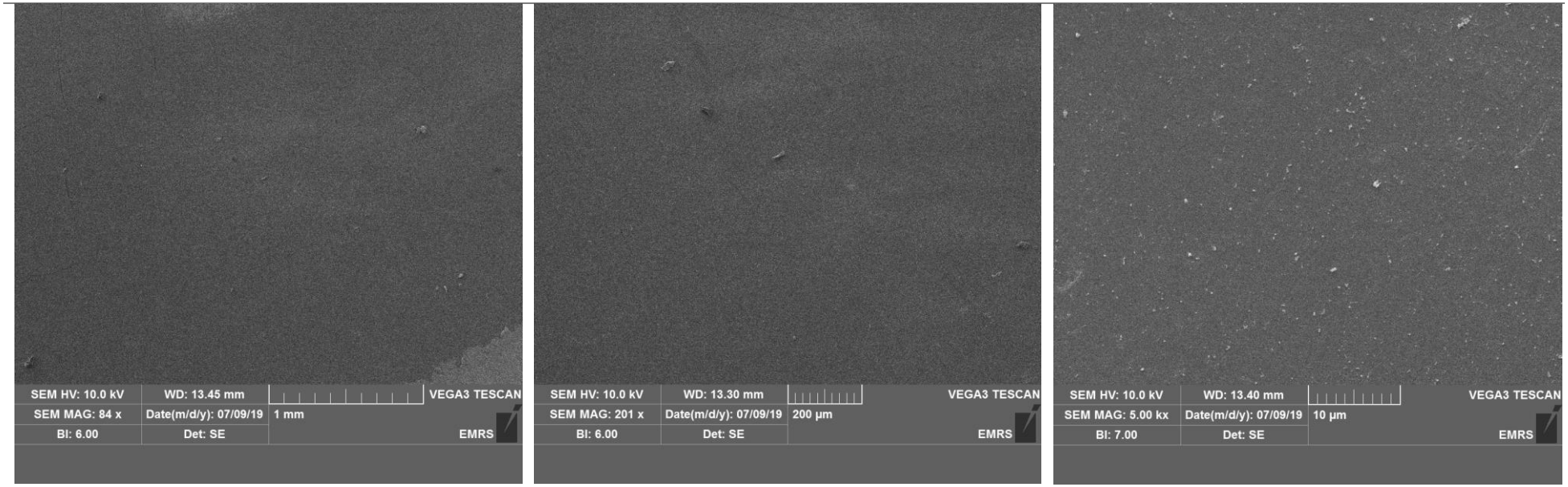


Figure 79 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP. No significant changes were noted apart from minor debris visible under high magnification.

6.6.2 Whitening using 6% Hydrogen Peroxide at pH7

Enamel surface topography

Enamel remineralised using CPP-ACP showed an overall smooth and un-affected surface, similar to the control enamel, with minor debris present as white spikes (Figure 80). Enamel treated with nHA, on the other hand, revealed significant surface irregularities.

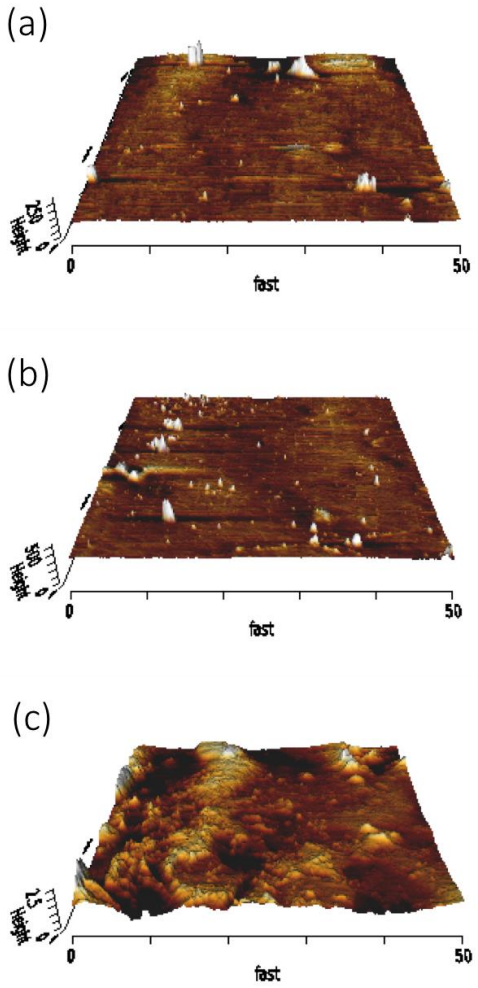


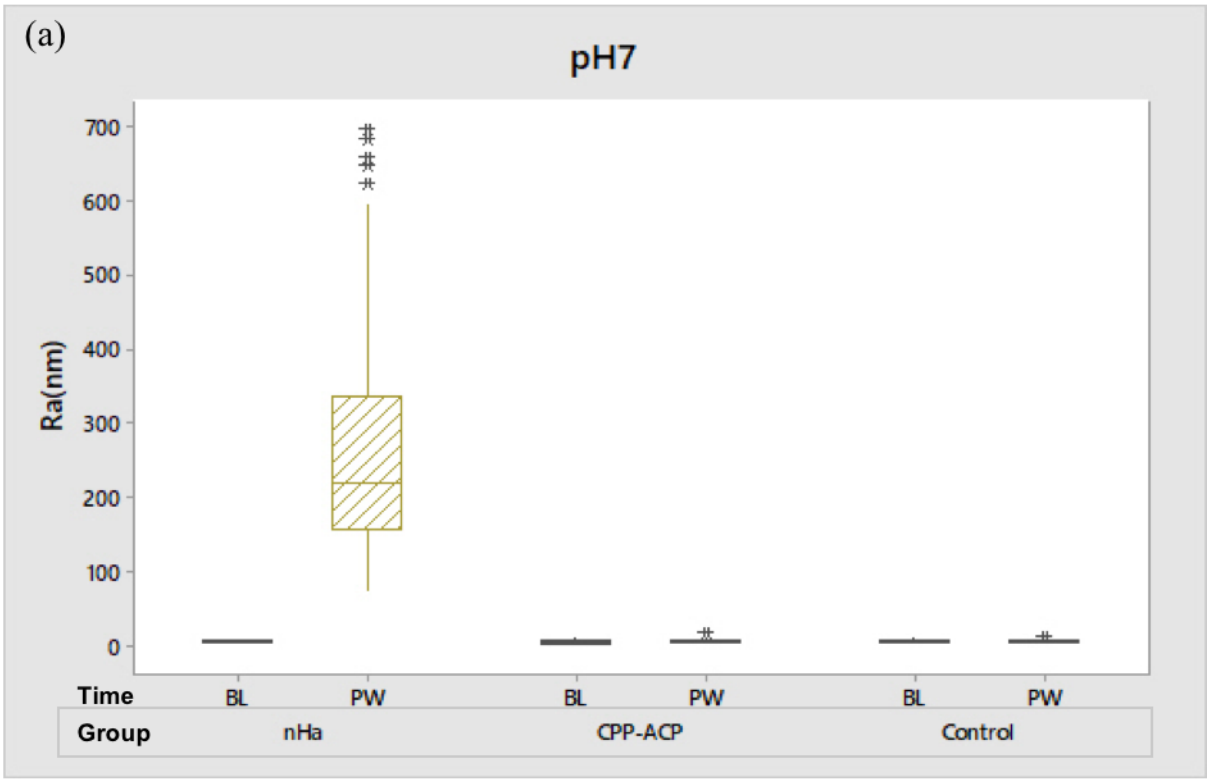
Figure 80 Representative 3D AFM images of whitened bovine enamel samples using 6% HP with a pH value of 7. Samples were divided into three groups; control (a), remineralised with CPP-ACP (b), and remineralised with nHA (c). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-b) is nm and in image (c) is µm.

Enamel roughness (Ra)

After remineralisation with nHA a significant increase in enamel Ra was recorded (P≤0.05) (Table 43) (Figure 81). Enamel remineralised using nHA had a significantly rougher surface in comparison to the control group and samples remineralised with CPP-ACP (P≤0.05). Enamel roughness was not significantly different between samples treated with CPP-ACP and control enamel samples (P>0.05).

	Ra (nm)	
Treatment	Before	After
nHA	7.7 (3.2)^{a,A}	220.9 (180.4)^{b,B}
CPP	5.9 (4.0)^{a,A}	6.7 (4.0)^{a,A}
Control	6.5 (3.8)^{a,A}	6.3 (2.8)^{a,A}

Table 43 Median (IQR) Ra values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.



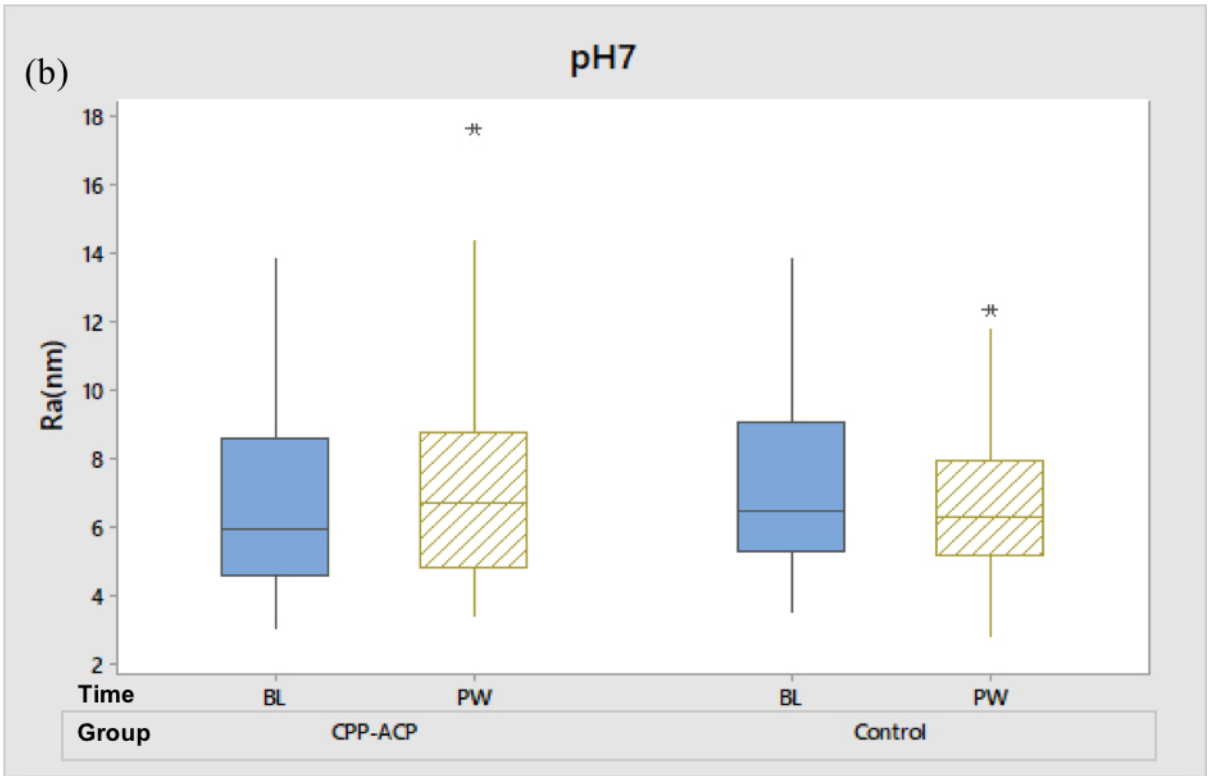


Figure 81 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group (a). Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using CPP-ACP in addition to a control group (b) Error bars represent the variability of data.

Enamel hardness (HV)

After remineralisation with nHA, a significant decrease in enamel Vickers hardness was recorded (P≤0.05) (Table 44) (Figure 82). Enamel hardness was significantly different between samples treated with CPP-ACP, nHA, and control enamel samples (P≤0.05).

	HV (H_{IT})	
Treatment	Before	After
nHA	276.8 (44.9)^{a,A}	207.4 (101.9)^{b,C}
CPP	275.0 (49.6)^{a,A}	298.1 (77.0)^{a,B}
Control	250.0 (26.0)^{a,A}	254.5 (19.5)^{a,A}

Table 44 Median (IQR) HV values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

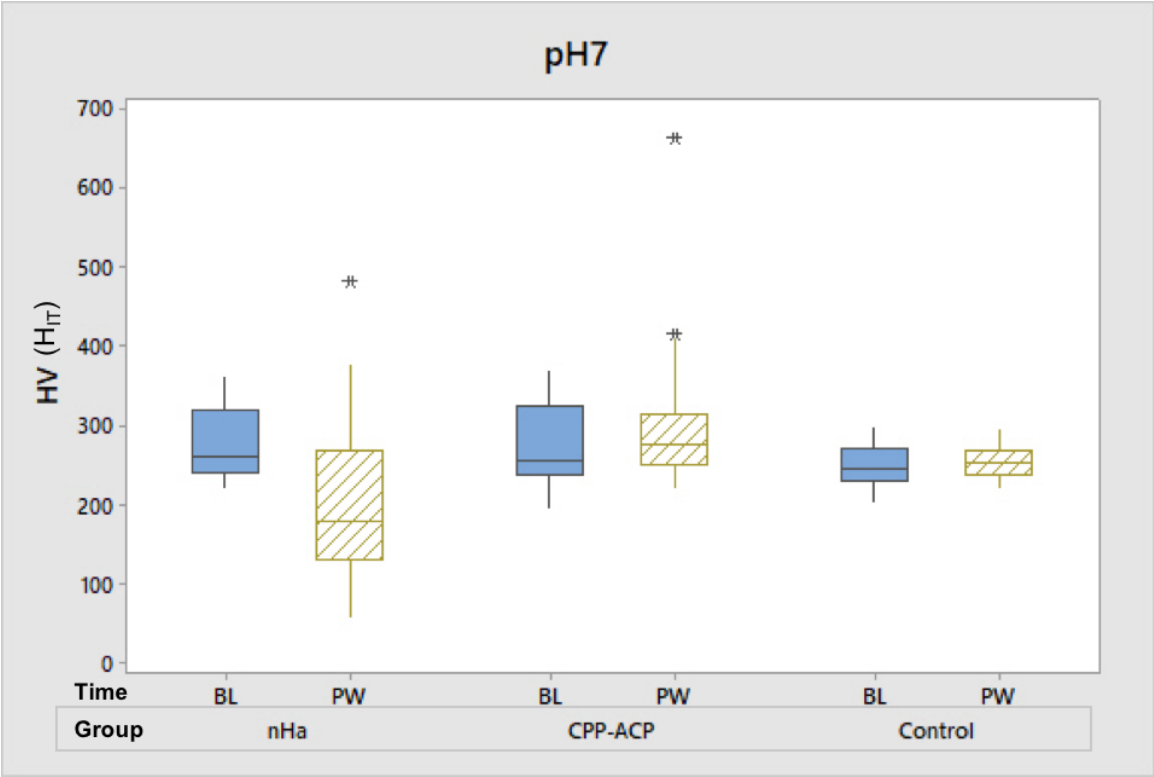


Figure 82 Box and whiskers plot of enamel HV at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group. Error bars represent the variability of data.

Colour change

Changes in colour (ΔE) were not significantly different between enamel remineralised with CPP-ACP and nHA, and control enamel samples (P>0.05) (Table 45). A significant increase in enamel lightness (L*) and decrease in b* values away from the yellow towards the blue spectrum were recorded in enamel treated with CPP-ACP, nHA, in addition to control enamel samples (P≤0.05). In addition, a significant reduction in a* values away from the red and towards the green spectrum occurred only in enamel remineralised with CPP-ACP (P≤0.05). Lightness values were significantly greater in enamel remineralised with CPP-ACP and nHA in comparison to control enamel (P≤0.05). In addition, reductions in b* values away from the yellow towards the blue spectrum were significantly greater in control enamel samples and enamel remineralised with CPP-ACP than samples treated with nHA (P≤0.05).

ΔE		
CPP-ACP	nHa	Control
11.6 (1.7)[a]	11.0 (2.0)[a]	11.7 (1.7)[a]
L* value		
	Before	After
CPP-ACP	80.4 (1.4)[a,A]	87.8 (0.9)[b,A]
nHA	80.8 (1.3)[a,A]	87.6 (0.6)[b,A]
Control	80.6 (2.2)[a,A]	85.9 (0.7)[b,B]
a* value		
	Before	After
CPP-ACP	-0.8 (0.4)[a,A]	-1.3 (0.1)[b,A,B]
nHA	-1.0 (0.4)[a,A]	-1.2 (0.1)[a,A]
Control	-1.0 (0.5)[a,A]	-1.4 (0.1)[a,B]
b* value		
	Before	After
CPP-ACP	11.1 (1.3)[a,A]	2.2 (0.6)[b,A]
nHA	11.7 (2.6)[a,A]	3.1 (1.0)[b,B]
Control	12.1 (1.3)[a,A]	1.8 (0.3)[b,A]

Table 45 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with CPP-ACP and nHA atomic % of enamel calcium, phosphorus, and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 46).

	Element	Atomic %
nHA	Ca	14.4 (4.5)
	P	32.8 (1.5)
	C	32.1 (11.9)
CPP-ACP	Ca	18.9 (0.8)
	P	29.7 (3.6)
	C	21.1 (1.8)
Control	Ca	11.3 (3.7)
	P	40.2 (0.6)
	C	45.9 (6.7)

Table 46 Mean atomic % values and standard deviation (SD) of calcium, phosphorous, and carbon in bovine enamel samples after whitening/remineralisation using CPP-ACP and nHA, in comparison to a control group.

Surface morphology

Enamel remineralised using nHA exhibited a rough irregular surface; caused by the accumulation of nHA crystals. Enamel treated with CPP-ACP showed a smooth surface, covered with what appears to be remnants of the remineralising agent. The control group showed an overall smooth surface with no visible damage (Figure 83-85).

nHA

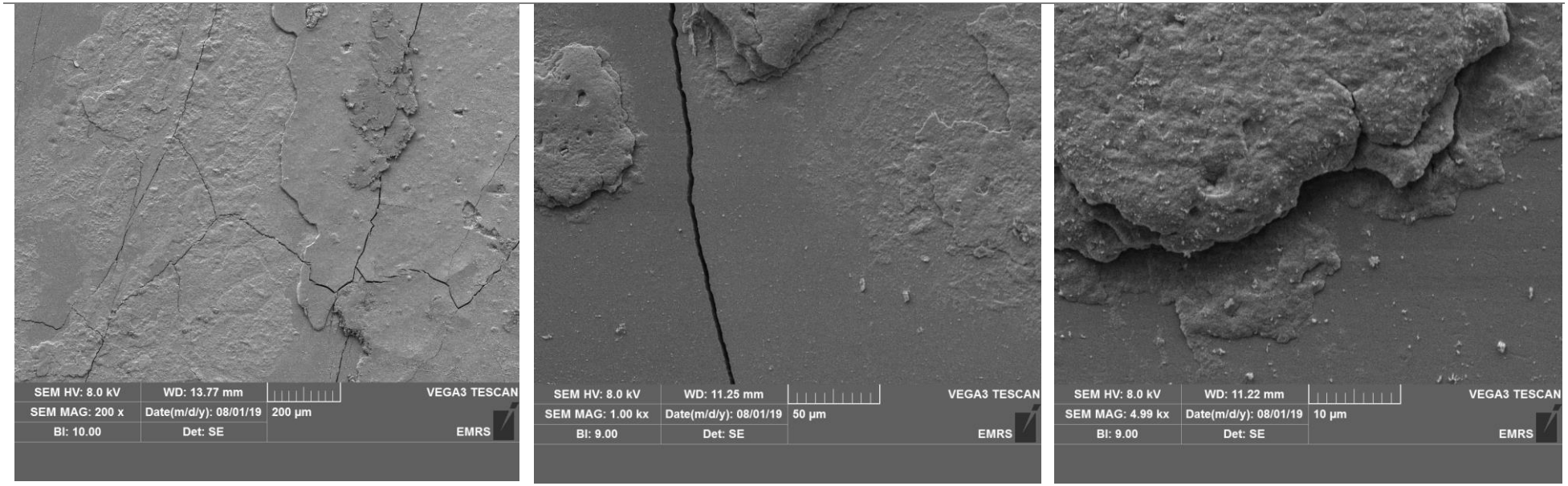


Figure 83 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP and remineralised using 15.5% nHA. Note the layer formed by the accumulation of nHA crystals.

CPP-ACP

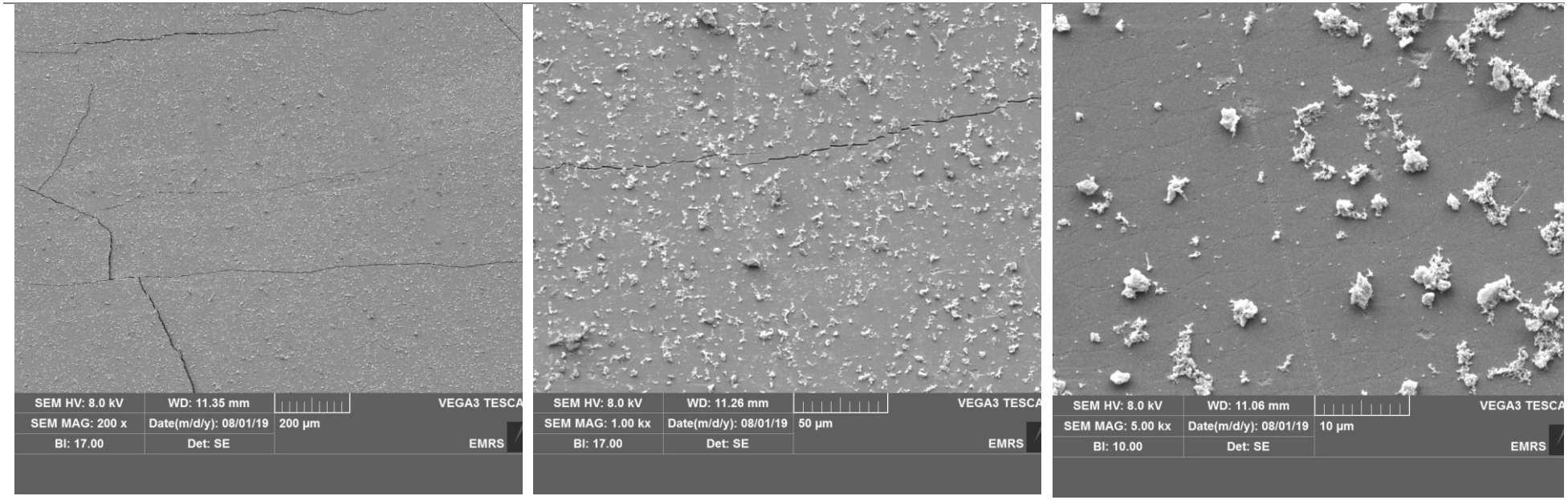


Figure 84 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP and remineralised using CPP-ACP. Note precipitants of CPP-ACP on the treated enamel surface.

Control

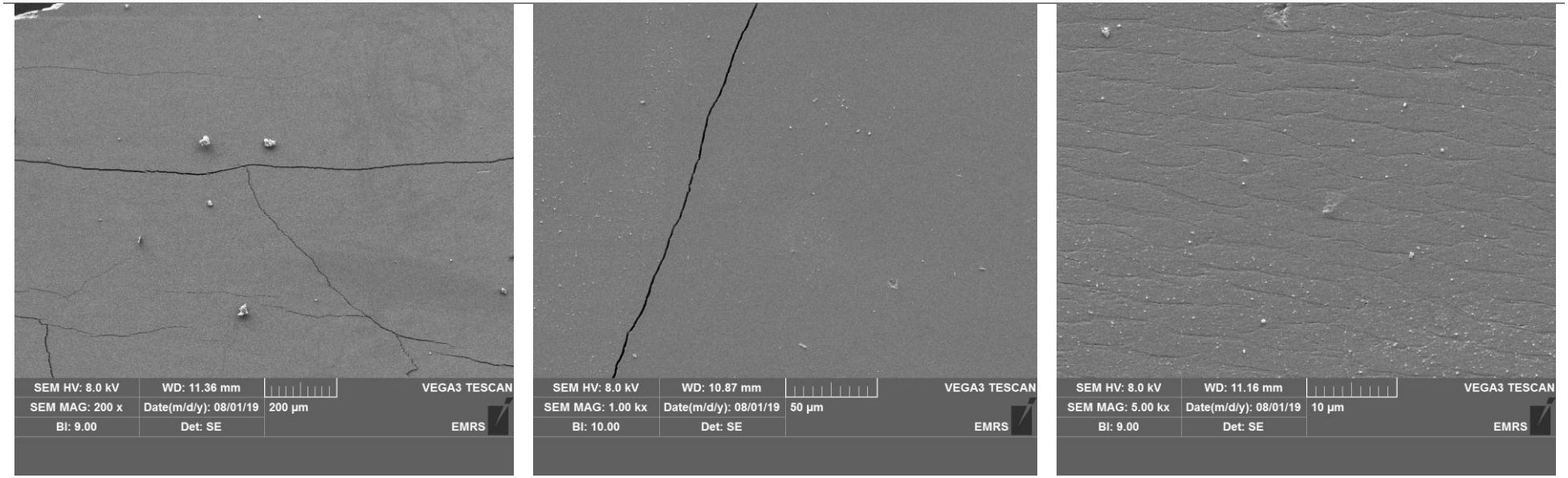


Figure 85 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP. No significant changes were noted apart from minor debris visible under high magnification.

6.6.3 Whitening using 6% Hydrogen Peroxide at pH9

Enamel surface topography

Enamel remineralised using CPP-ACP showed an overall smooth and un-affected surface, similar to the control enamel, with minor debris present as white spikes (Figure 86). Enamel treated with nHA, on the other hand, revealed significant surface irregularities visible as sharp peaks and deep valleys randomly distributed across the treated surface.

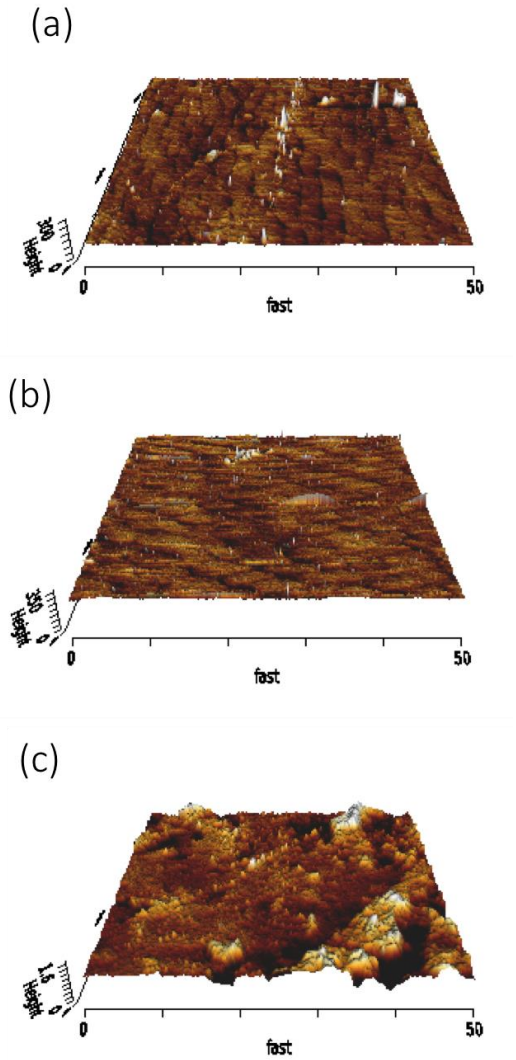


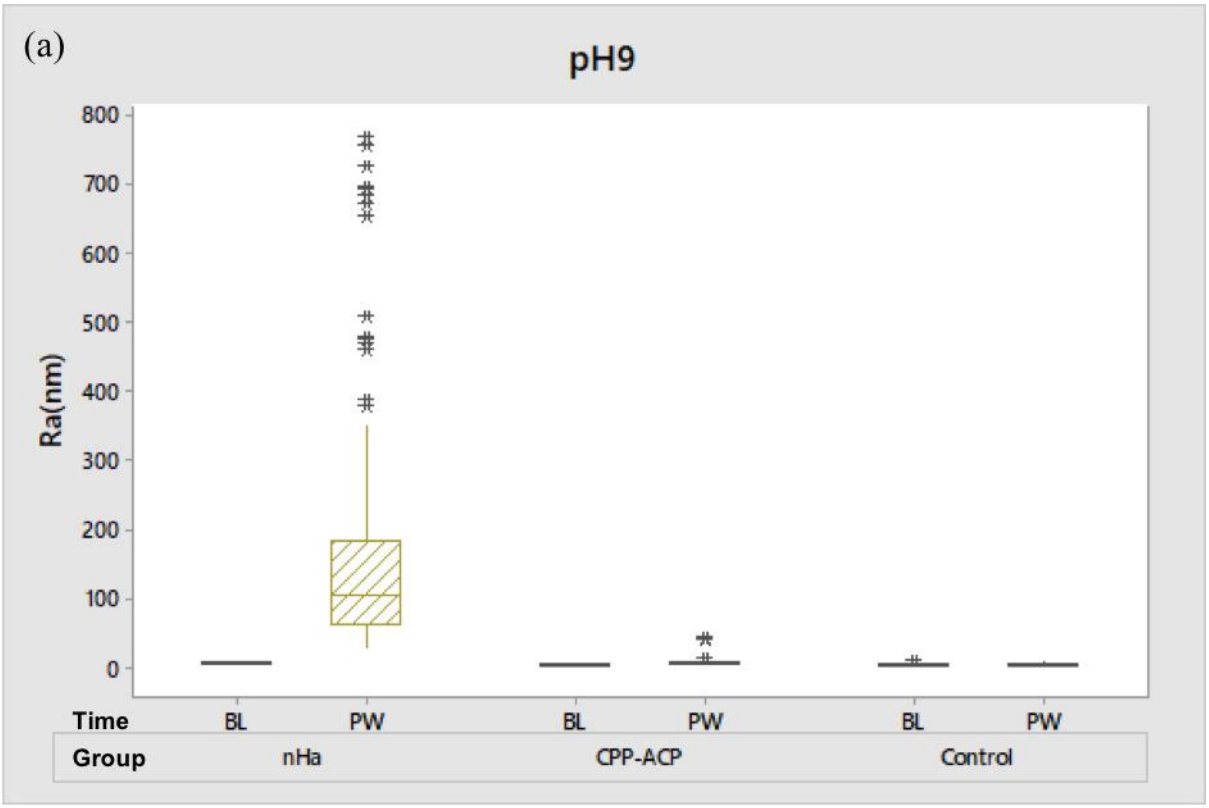
Figure 86 Representative 3D AFM images of whitened bovine enamel samples using 6% HP with a pH value of 9. Samples were divided into three groups; control (a), remineralised with CPP-ACP (b), and remineralised with nHA (c). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-scale unit in images (a-b) is nm and in image (c) is µm.

Enamel roughness (Ra)

After remineralisation with CPP-ACP and nHA a significant increase in enamel Ra was recorded (P≤0.05) (Table 47) (Figure 87). In addition, control enamel samples showed a significant increase in roughness after treatment (P≤0.05). Enamel remineralised using nHA had a significantly rougher surface in comparison to the control group and samples treated with CPP-ACP (P≤0.05). In addition, enamel roughness was significantly greater in samples treated with CPP-ACP in comparison to control enamel samples (P≤0.05).

	Ra (nm)	
Treatment	Before	After
nHA	7.1 (3.6)^{a,A}	105.8 (121.8)^{b,C}
CPP	5.5 (3.0)^{a,A}	8.3 (2.9)^{b,B}
Control	5.4 (2.6)^{a,A}	6.2 (3.1)^{b,A}

Table 47 Median (IQR) Ra values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.



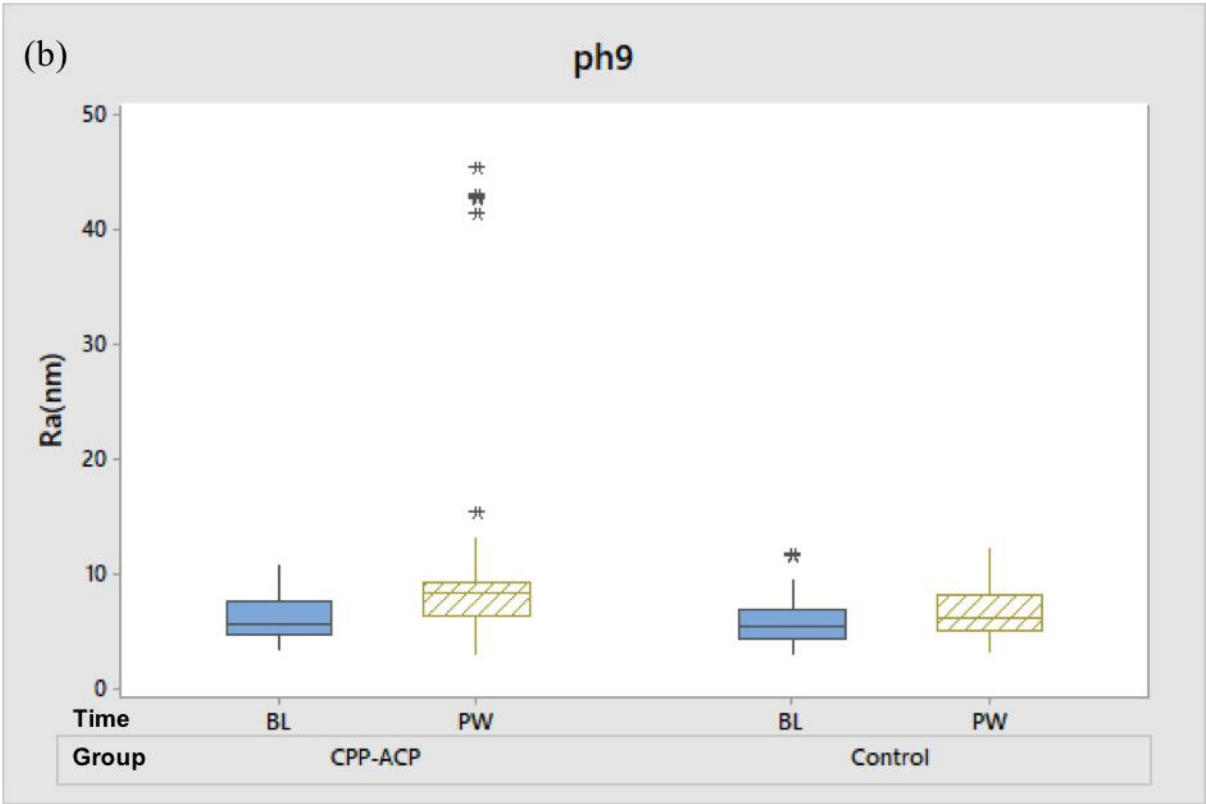


Figure 87 Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group (a). Box and whiskers plot of enamel Ra at baseline (BL) and post whitening (PW)/ remineralisation using CPP-ACP in addition to a control group (b) Error bars represent the variability of data.

Enamel hardness (HV)

After remineralisation with nHA, a significant decrease in enamel Vickers hardness was recorded (P≤0.05) (Table 48) (Figure 88). Enamel HV did not significantly change in the control group and in samples remineralised with CPP-ACP (P>0.05). Enamel hardness was significantly lower in samples treated with nHA in comparison to control enamel samples and enamel remineralised with CPP-ACP (P≤0.05).

	HV (H_{IT})	
Treatment	Before	After
nHA	272.5 (28.6)^{a,A}	141.5 (98.5)^{b,B}
CPP	262.0 (32.4)^{a,A}	257.1 (49.2)^{a,A}
Control	272.8 (29.2)^{a,A}	277.2 (39.5)^{a,A}

Table 48 Median (IQR) HV values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

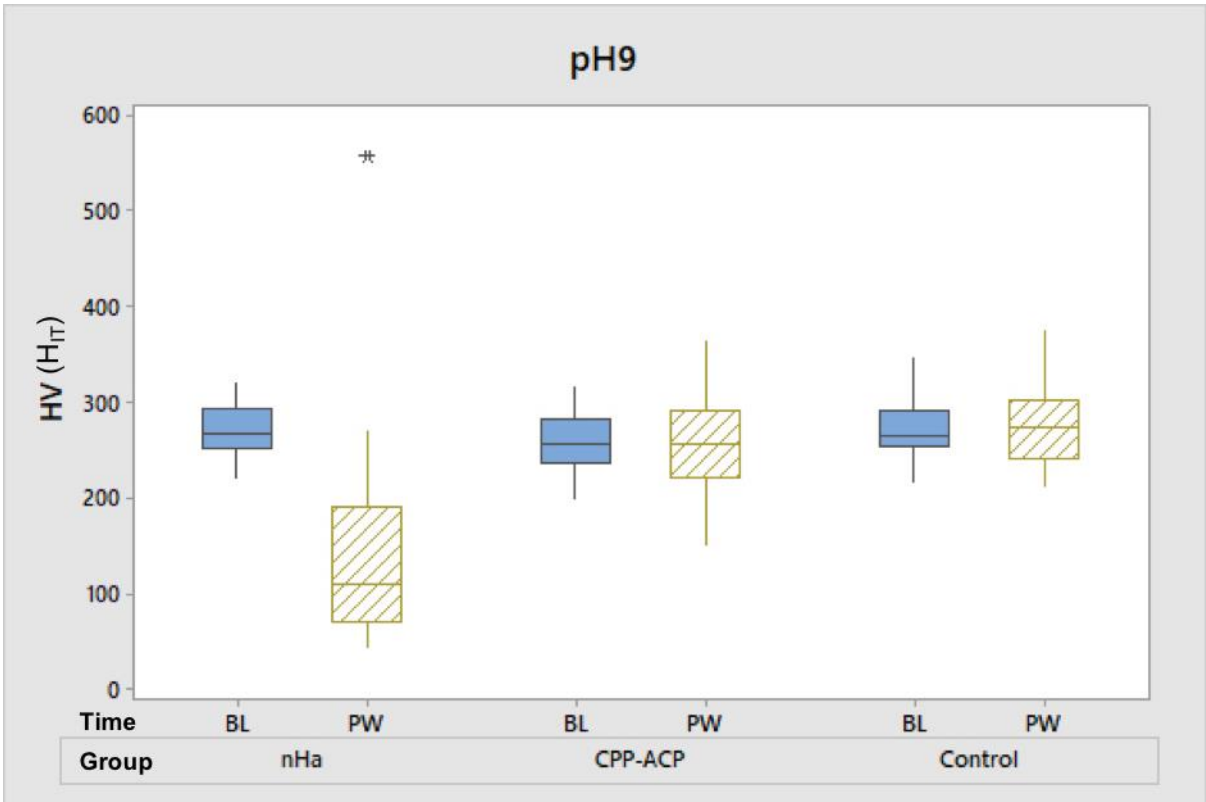


Figure 88 Box and whiskers plot of enamel HV at baseline (BL) and post whitening (PW)/ remineralisation using nHA, CPP-ACP, in addition to a control group. Error bars represent the variability of data.

Colour change

Changes in colour (ΔE) were not significantly different between enamel remineralised with CPP-ACP and nHA, and control enamel samples (P>0.05) (Table 49). A significant increase in enamel lightness (L*) and decrease in (b*) values away from the yellow towards the blue spectrum were recorded in enamel treated with CPP-ACP and nHA, in addition to control enamel samples (P≤0.05). In addition, a significant increase in (a*) values away from the green and towards the red spectrum only occurred in control enamel samples (P≤0.05). Lightness (L*) and (a*) values were not significantly different between enamel remineralised with CPP-ACP and nHA, and control enamel samples (P>0.05). In addition, reductions in (b*) values away from the yellow towards the blue spectrum were significantly greater in control enamel samples and enamel remineralised with CPP-ACP than samples treated with nHA (P≤0.05).

ΔE		
CPP-ACP	nHa	Control
10.2 (1.4)^{a}	9.3 (1.6)^{a}	10.7 (1.2)^{a}
L* value		
	Before	After
CPP-ACP	82.5 (1.8)^{a,A}	88.1 (0.8)^{b,A}
nHa	83.1 (1.6)^{a,A}	88.8 (1.2)^{b,A}
Control	82.2 (1.0)^{a,A}	88.5 (1.1)^{b,A}
a* value		
	Before	After
CPP-ACP	-1.7 (0.5)^{a,A}	-1.4 (0.1)^{a,A}
nHa	-1.6 (0.4)^{a,A}	-1.4 (0.1)^{a,A}
Control	-1.7 (0.4)^{a,A}	-1.4 (0.1)^{b,A}
b* value		
	Before	After
CPP-ACP	8.8 (5.0)^{a,A}	2.6 (0.7)^{b,A}
nHa	10.5 (1.4)^{a,A}	3.6 (1.1)^{b,B}
Control	11.0 (1.3)^{a,A}	2.5 (0.6)^{b,A}

Table 49 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of bovine enamel samples before and after whitening/remineralisation using CPP-ACP and nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with CPP-ACP and nHA atomic % of enamel calcium, phosphorus, and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 50).

	Element	Atomic %
nHA	Ca	19.6 (0.2)
	P	19.9 (0.1)
	C	41.3 (1.9)
CPP-ACP	Ca	28.9 (2.1)
	P	22.4 (1.2)
	C	36.7 (1.8)
Control	Ca	24.9 (4.5)
	P	20.0 (2.0)
	C	35.4 (0.1)

Table 50 Mean atomic % values and standard deviation (SD) of calcium, phosphorous, and carbon in bovine enamel samples after whitening/remineralisation using CPP-ACP and nHA, in comparison to a control group.

Surface morphology

Enamel remineralised using nHA exhibited a rough irregular surface; caused by the accumulation of nHA crystals and HP pH. Enamel treated with CPP-ACP showed a smooth surface with no visible damage. Control enamel, on the other hand, showed distinct surface irregularities and deformation of prismatic and inter-prismatic enamel (Figure 89-91).

nHA

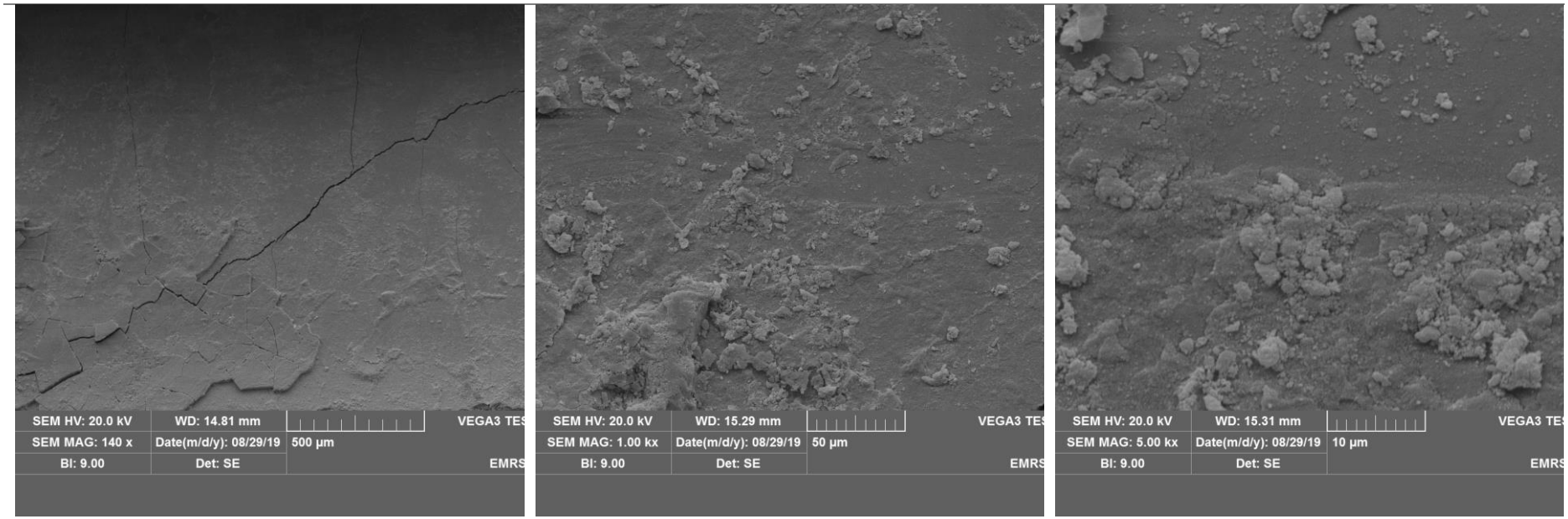


Figure 89 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP and remineralised using 15.5% nHA. Note the layer formed by the accumulation of nHA crystals in addition to the surface deformation caused by the solution pH.

CPP-ACP

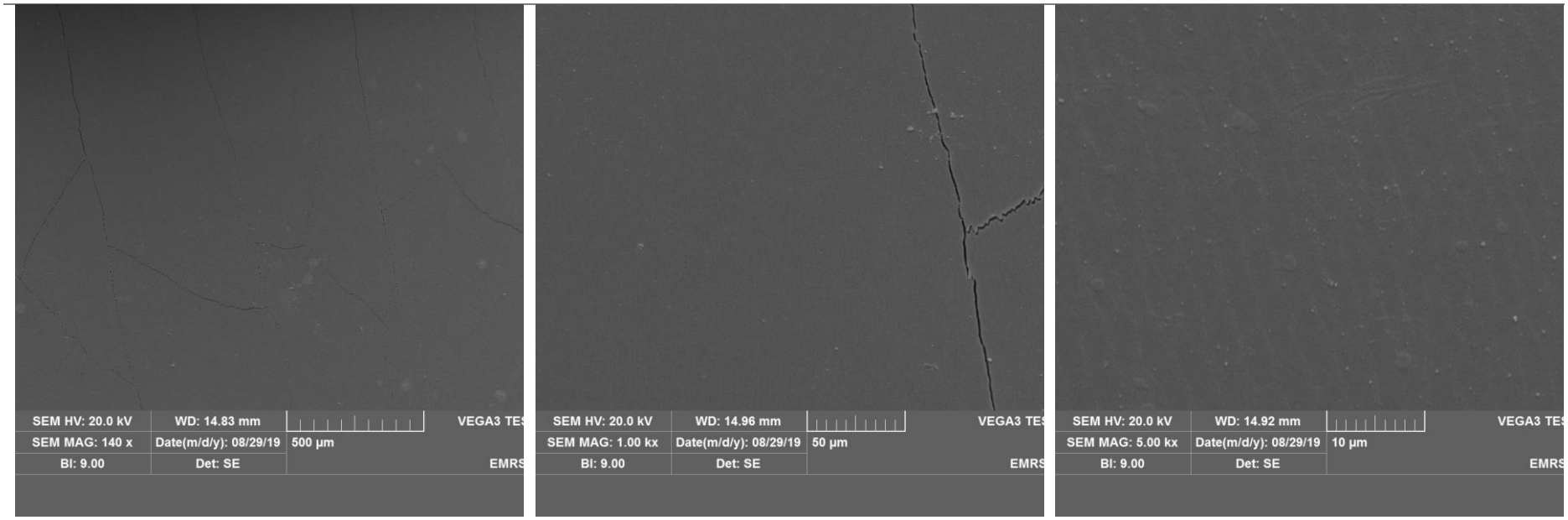


Figure 90 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP and remineralised using CPP-ACP. No significant changes were noted in treated enamel surfaces.

Control

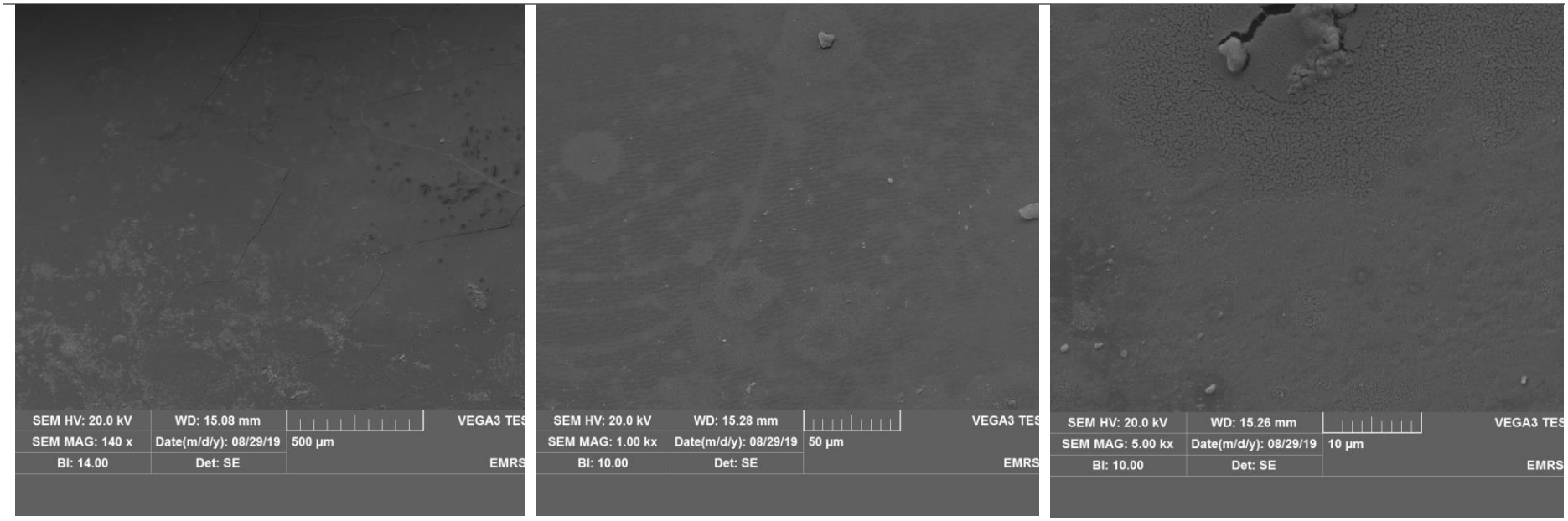


Figure 91 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP. Note the distinct surface irregularities and deformation of prismatic and inter-prismatic enamel.

6.6.4 Treatment using PBS (Control)

Enamel treated with CPP-ACP showed minor surface irregularities, while control enamel samples had an overall smooth and un-affected surface (Figure 92). Enamel treated with nHA, on the other hand, revealed significant surface irregularities visible as sharp peaks and deep valleys randomly distributed across the treated surface.

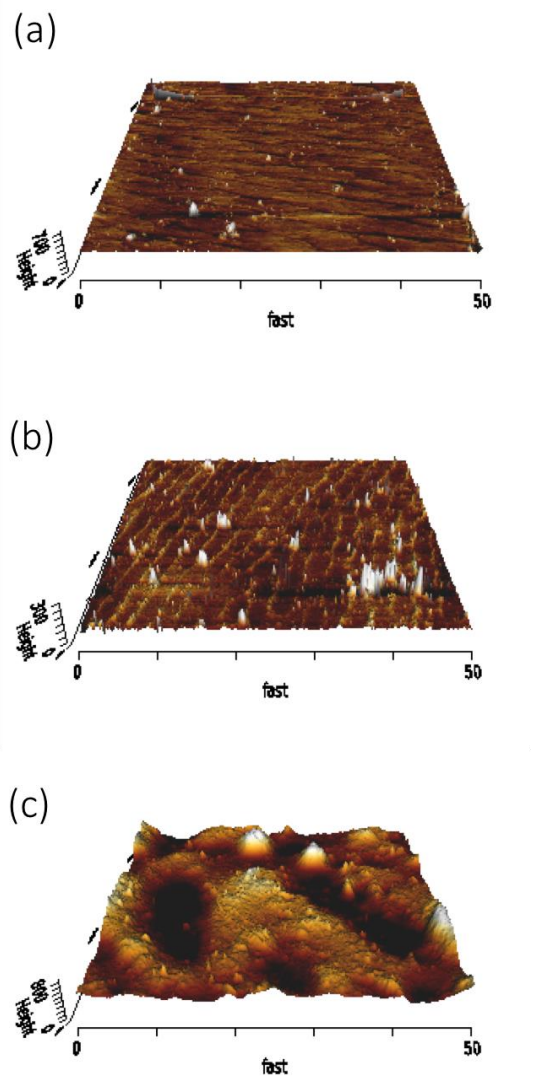


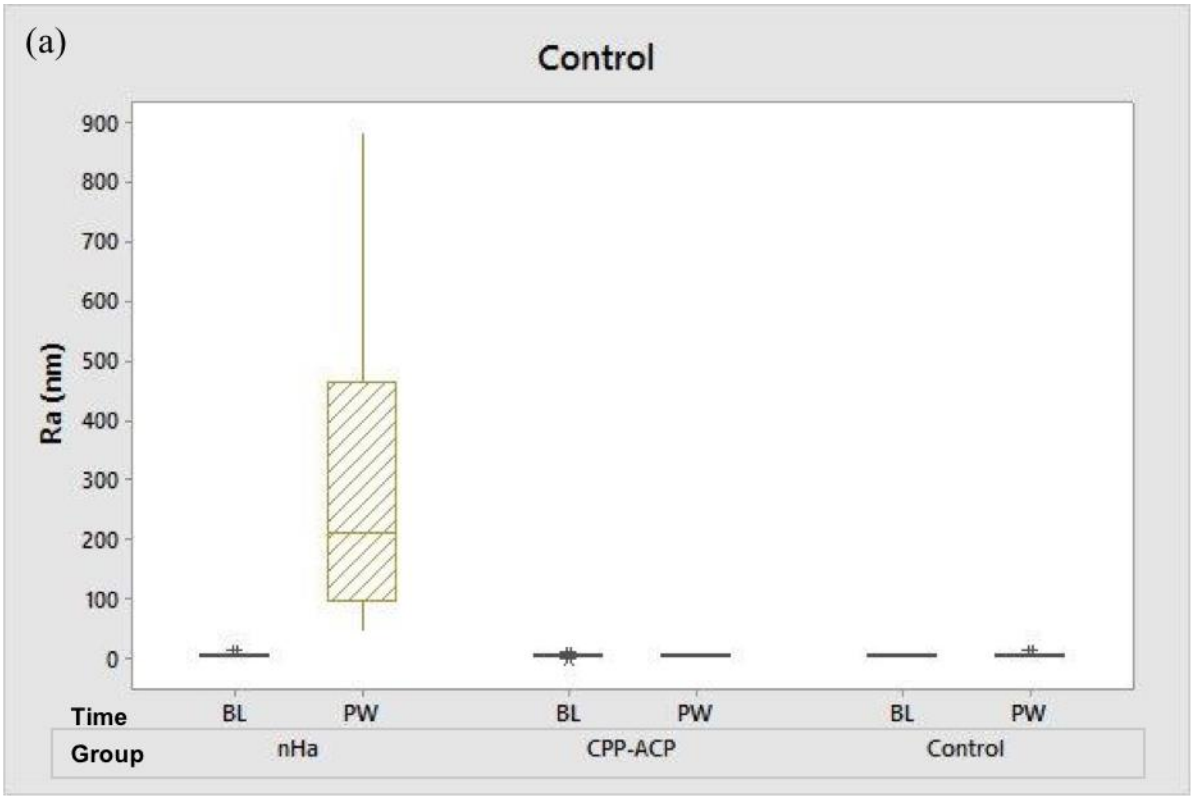
Figure 92 Representative 3D AFM images of bovine enamel samples treated with PBS. Samples were divided into three groups; control (a), treated with CPP-ACP (b), and treated with nHA (c). Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed.

Enamel roughness (Ra)

After treatment with nHA a significant increase in enamel Ra was recorded (P≤0.05) (Table 51) (Figure 93). In addition, control enamel samples showed a significant increase in roughness after treatment (P≤0.05). Enamel treated with nHA had a significantly rougher surface in comparison to control enamel samples and samples treated with CPP-ACP (P≤0.05).

	Ra (nm)	
Treatment	Before	After
nHA	6.8 (3.4)^{a,A}	211.3 (366.5)^{b,B}
CPP	6.7 (1.8)^{a,A}	6.7 (3.4)^{aA}
Control	5.4 (2.9)^{a,A}	6.6 (3.2)^{b,A}

Table 51 Median (IQR) Ra values of bovine enamel samples before and after treatment using PBS/CPP-ACP, nHA, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.



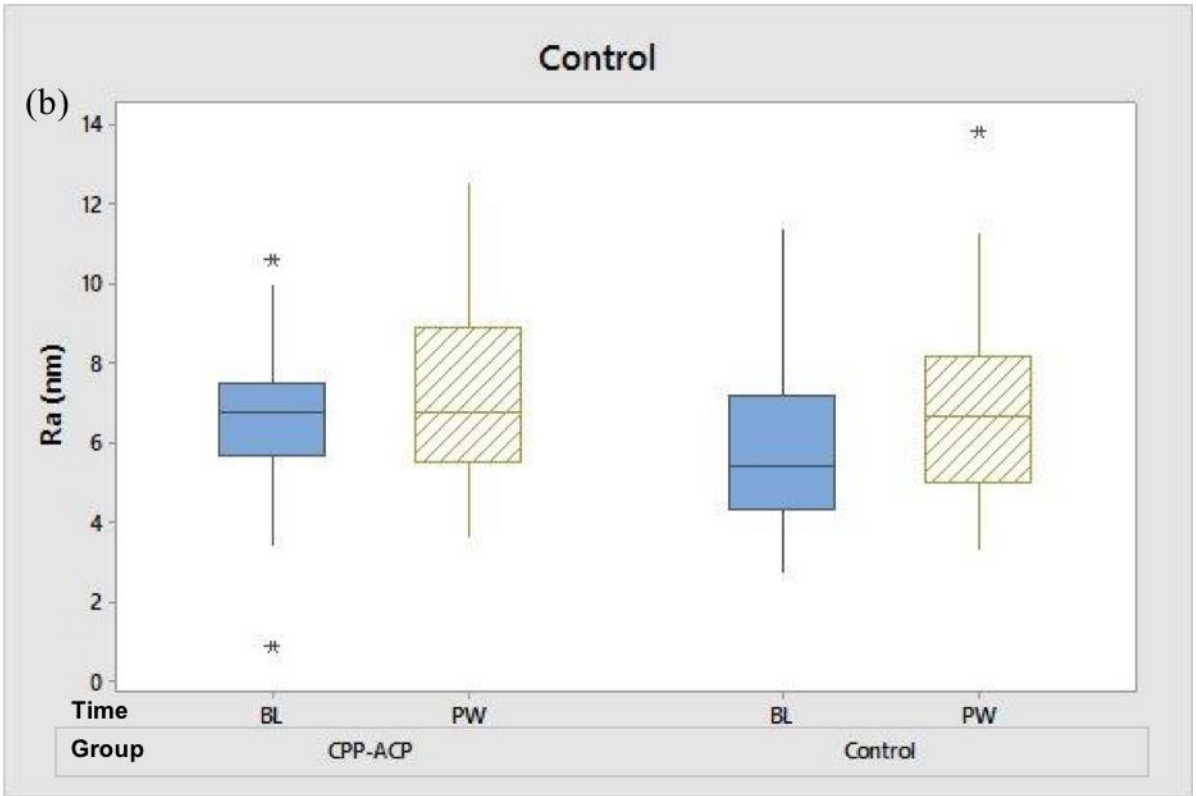


Figure 93 Box and whiskers plot of enamel Ra at baseline (BL) and post treatment (PW) using PBS/ nHA, CPP-ACP, in addition to a control group (a). Box and whiskers plot of enamel Ra at baseline (BL) and post treatment (PW) using PBS/ CPP-ACP in addition to a control group (b) Error bars represent the variability of data.

Enamel hardness (HV)

After treatment with nHA and CPP-ACP, a significant decrease in enamel Vickers hardness was recorded (P≤0.05) (Table 52) (Figure 94). Enamel hardness was significantly lower in samples treated with nHA in comparison to control enamel samples and enamel treated with CPP-ACP (P≤0.05).

	HV (H_{IT})	
Treatment	Before	After
nHA	285.6 (109.9)^{a,A}	110.0 (131.5)^{b,B}
CPP	275.9 (52.0)^{a,A}	237.2 (59.4)^{b,A}
Control	253.2 (77.3)^{a,A}	275.0 (58.1)^{a,A}

Table 52 Median (IQR) HV values of bovine enamel samples before and after treatment using PBS/ nHA, CPP-ACP, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.

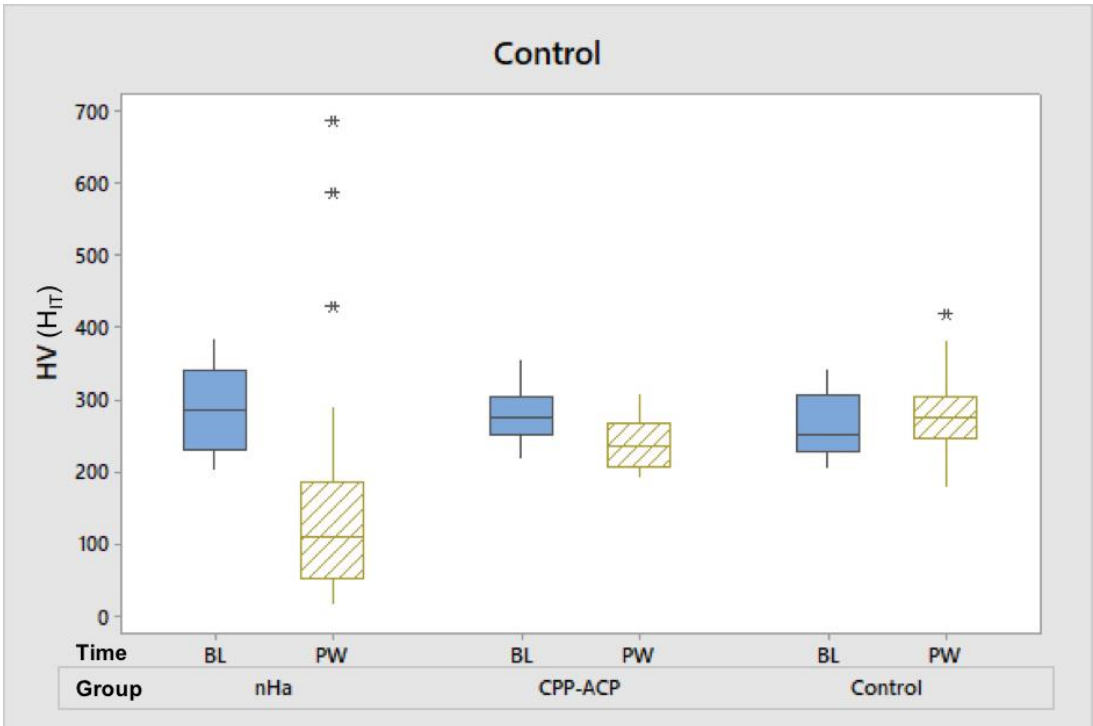


Figure 94 Box and whiskers plot of enamel HV at baseline (BL) and post treatment (PW)using PBS/ nHA, CPP-ACP, in addition to a control group. Error bars represent the variability of data.

Colour change

Changes in colour (ΔE) were not significantly different between enamel treated with CPP-ACP and nHA, and control enamel samples (P>0.05) (Table 53). A significant decrease in enamel lightness (L*) and increase in (a*) values away from the green and towards the red spectrum occurred in all treated enamel samples (P≤0.05). In addition, no significant changes occurred in (b*) values along the yellow-blue spectrum in enamel treated with CPP-ACP and nHA, and control enamel samples (P>0.05). After treatment, no significant differences were recorded between groups along the (L*), (a*), and (b*) values (P>0.05).

ΔE		
CPP-ACP	nHa	Control
3.9 (2.2)[a]	3.3 (0.8)[a]	4.9 (1.3)[a]
L* value		
	Before	After
CPP-ACP	82.8 (2.0)[a,A]	79.4 (1.4)[b,A]
nHA	81.2 (2.1)[a,A]	79.1 (1.1)[b,A]
Control	83.1 (1.8)[a,A]	78.9 (1.2)[b,A]
a* value		
	Before	After
CPP-ACP	-0.6 (0.3)[a,A]	0.1 (0.9)[b,A]
nHA	-1.0 (0.6)[a,A]	-0.1 (0.8)[b,A]
Control	-0.2 (0.3)[a,A]	0.6 (0.5)[b,A]
b* value		
	Before	After
CPP-ACP	9.7 (1.5)[a,A]	8.8 (1.3)[a,A]
nHA	7.0 (2.2)[a,A]	8.4 (1.3)[a,A]
Control	10.1 (1.8)[a,A]	9.6 (1.3)[a,A]

Table 53 The mean and standard deviation (SD) of ΔE, L, a*, and b* values of bovine enamel samples before and after treatment using PBS/ nHA, CPP-ACP, in addition to a control group. Different lowercase superscript letters indicate significant differences between columns and different uppercase superscript letters indicate significant differences between rows.*

Mineral composition

After treatment with CPP-ACP and nHA atomic % of enamel calcium, phosphorus, and carbon were not significantly different than the those recorded in the control group (P>0.05) (Table 54).

	Element	Atomic %
nHa	Ca	30.4 (0.1)
	P	24.7 (3.0)
	C	10.6 (1.2)
CPP-ACP	Ca	33.5 (4.5)
	P	36.0 (5.0)
	C	18.1 (2.3)
Control	Ca	40.8 (3.2)
	P	33.6 (8.0)
	C	11.1 (3.6)

Table 54 Mean atomic % values and standard deviation (SD) of calcium, phosphorous, and carbon in bovine enamel samples after whitening/remineralisation using CPP-ACP and nHA, in comparison to a control group.

Surface morphology

Enamel treated with nHA exhibited a rough irregular surface; caused by the accumulation of nHA crystals. Enamel treated with CPP-ACP and control enamel samples, on the other hand, showed an overall smooth surface with no visible damage (Figure 95-97).

nHA

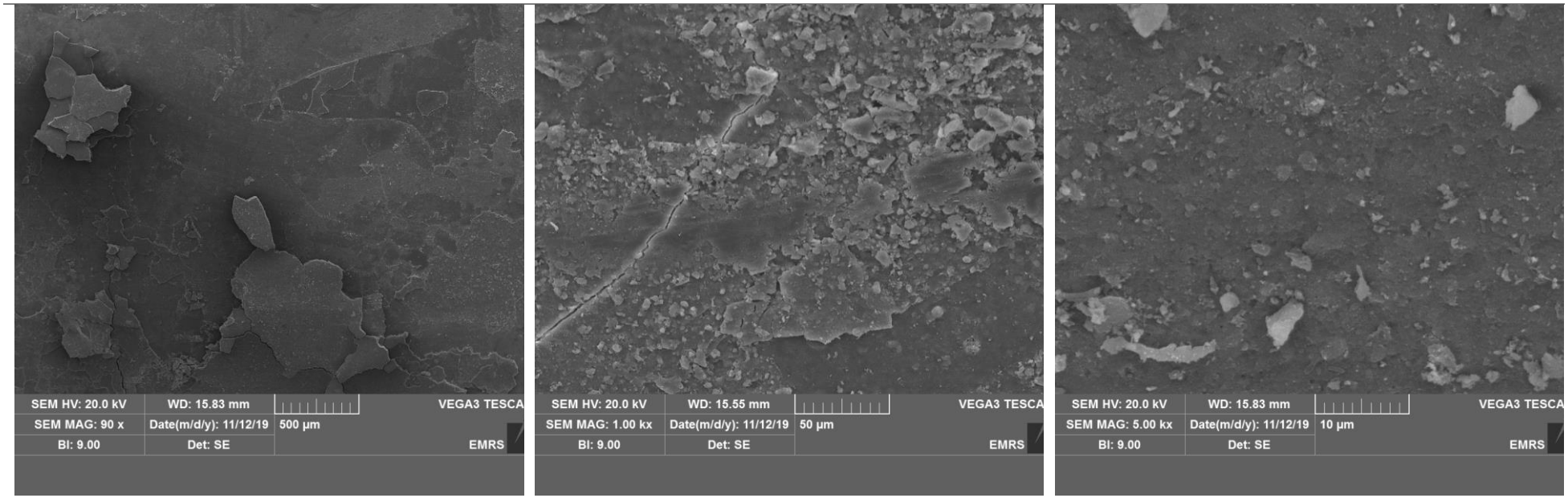


Figure 95 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS and 15.5% nHA. Note the layer formed by the accumulation of nHA crystals.

CPP-ACP

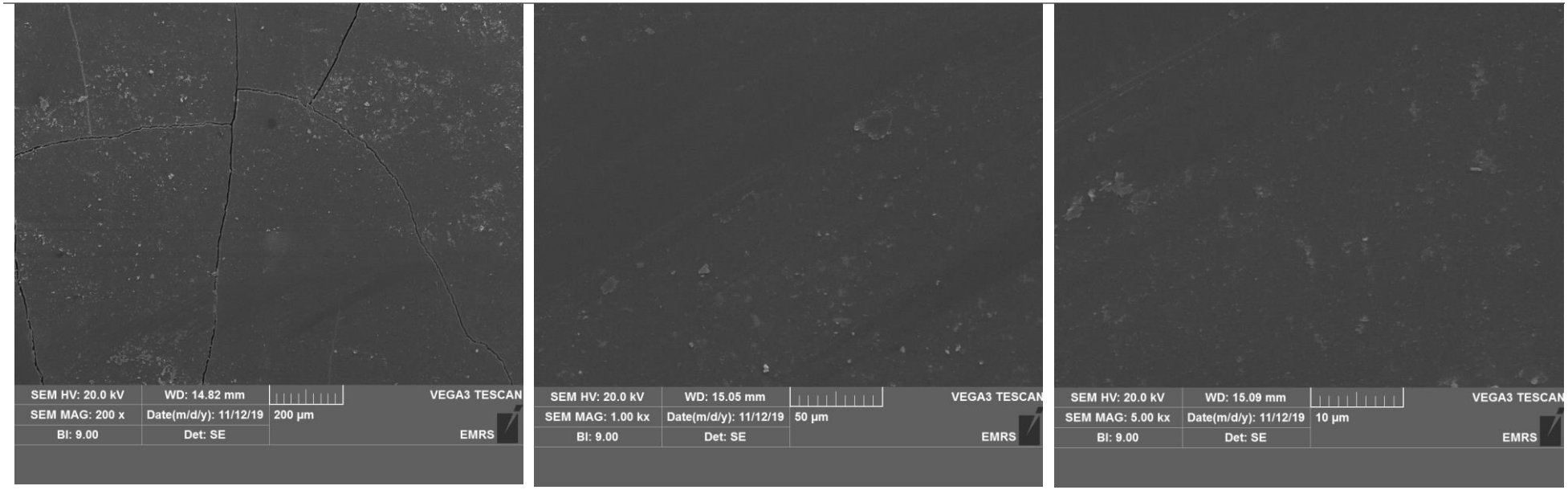


Figure 96 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS and CPP-ACP. No significant changes were noted in treated enamel surfaces apart from minor debris visible under low magnification.

Control

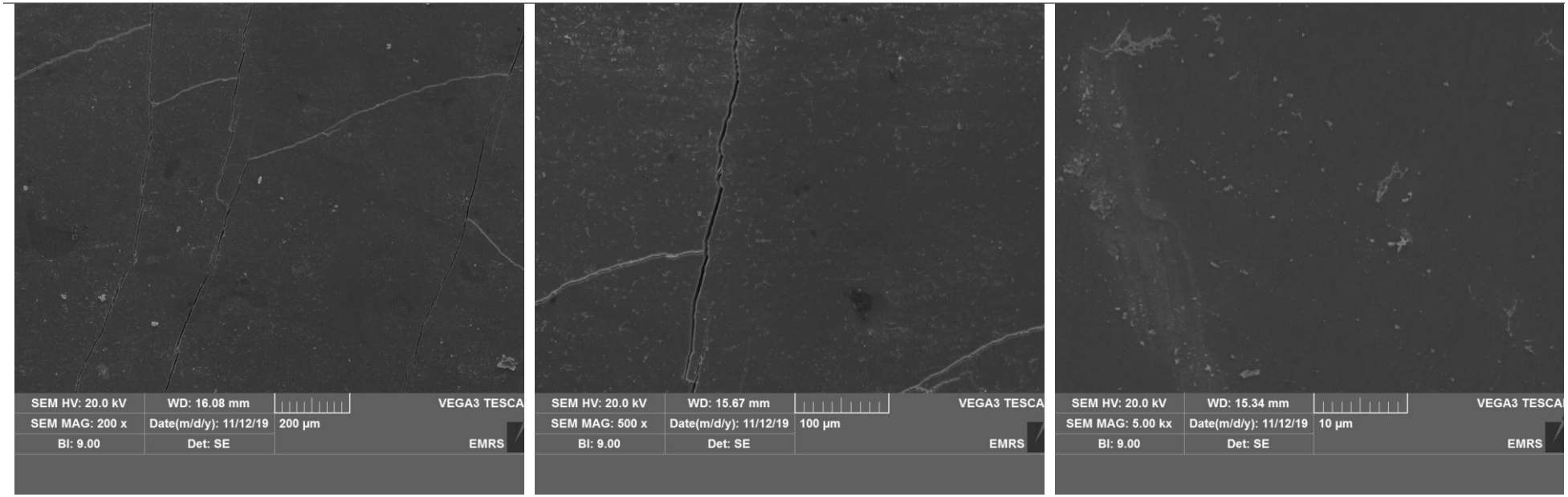


Figure 97 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS. No significant changes were noted in treated enamel surfaces apart from minor debris visible under all magnifications.

6.7 Discussion

Remineralising agents play an important role in restoring enamel to pre-whitened conditions and in stabilising the ionic levels of calcium and phosphate (Bayrak *et al.*, 2009). Historically, the most commonly used remineralising agent is fluoride, which has been extensively studied, tested, and modified in an attempt to optimise its biological effectiveness in preventing dental caries (Murray *et al.*, 1991; Swarup and Rao, 2012; Kanduti *et al.*, 2016; Walsh *et al.*, 2019). It is worth noting, however, that fluoride minimises apatite dissolution by binding with calcium and phosphate, resulting in the formation of fluorapatite as opposed to restoring lost apatite minerals, which highlights its preventive rather than regenerative capacity (Swarup and Rao, 2012).

New more promising materials are being discovered and investigated such as CPP-ACP and nHA which were selected as part of this current research as remineralising agents reported to replace and restore lost enamel apatite. As mentioned earlier in Chapter 2, nHA is one of the most biocompatible and bioactive materials due to its similarity to dental apatite and its potential to restore dental enamel (Kim *et al.*, 2007; Huang *et al.*, 2010; Juntavee *et al.*, 2018). The size and concentration of nHA crystals chosen in this study are <50nm and 15.5% respectively, which are in line with reported apatite crystal size ranging from 20-40nm and previously tested nHA concentrations ranging from 1% to 15% (Kim *et al.*, 2007; Huang *et al.*, 2009; Swarup and Rao, 2012). Since the nHA paste used (NanoXIM CarePaste™, Fluidinova, Moreira da Maia, Portugal) was not a commercially available product, the application time was set to follow the same application duration recommended for GC tooth mousse (Recaldent™, Henry Schein Laboratory, Gillingham, UK). The CPP-ACP based product applied is a popular remineralising agent recommended for clinical use to restore and remineralise enamel (Asokan *et al.*, 2019). Differences in their impact on whitened enamel colour, Vickers hardness, roughness average, mineral composition, and surface microstructure will be discussed below.

6.7.1 Enamel roughness (Ra)

In an attempt to minimise the increase in enamel roughness as a result of being exposed to HP, the whitening protocol which included different pH values of 6% HP was combined with remineralising agents. This was a novel approach to develop maximum change in colour with minimal change in roughness. According to current results the application of nHA created a rougher enamel surface, irrespective of the HP pH tested. In addition, enamel treated with nHA were significantly rougher in comparison to other treatment groups. This was caused by the aggregation of nano-crystals on the whitened enamel surface (Rezvani *et al.*, 2015) by the continuous application of nHA paste after each whitening cycle, creating an irregular fragile

layer. After applying nHA, the nano-crystals adhere to enamel defects caused by the whitening process (Swarup and Rao, 2012). The crystals then start to aggregate, forming micro-clusters which cover the entire surfaces of prismatic and interprismatic enamel. The use of 15.5% nHA in this study caused nano-crystals to visibly cluster on the whitened enamel surface, forming a thin irregular layer (Figure 98). This could be explained by the low solubility of hydroxyapatite which at greater concentrations an increase in the deposition rate and quantity are evident (Huang *et al.*, 2009). This in turn prevents nanocrystals from penetrating deep into enamel micro and nano-cracks by blocking the surface pores and restricting access to deeper demineralised enamel. Therefore, although 15% nHA did show good remineralising effects according to the literature, crystal aggregation was unavoidable (Huang *et al.*, 2009). Previously published results regarding the remineralising effects of nHA diverge, as some claim that nHA causes a slight increase in whitened enamel roughness (Hassan *et al.*, 2016), while others claim that nHA significantly reduces enamel roughness post treatment (Selivany and Al-Hano, 2015). According to Hassan *et al.*, the increase in enamel roughness as a result of the application of nHA is caused by the precipitation and accumulation of nano-crystals; forming an irregular apatite surface (Hassan *et al.*, 2016). Differences between reported results concerning the effects of nHA on whitened enamel roughness are mostly attributed to the agent concentration, application technique, application time, and form of the remineralising agent used (Selivany and Al-Hano, 2015).

Roughness values in enamel treated with pH7 HP and PBS (control) then remineralised using CPP-ACP did not significantly change or differ from their respective control groups, where no remineralising agents were used post-whitening/treatment. On the other hand, the application of CPP-ACP caused a significant increase in roughness in samples whitened using pH5 HP. Enamel whitened using pH5 HP then treated with CPP-ACP had a statistically significant increase in Ra values post treatment, although not significantly different from Ra values obtained from the control group. Ion release from CPP-ACP was reported to rapidly increase in a neutral environment in comparison to an acidic environment (Limeback *et al.*, 2012). Therefore, applying CPP-ACP for 5 minutes immediately after whitening using a pH5 HP solution might have slowed the breakdown of the remineralising agent, and hence; limiting its benefits. This is in accordance to a study conducted by Chuna *et al*., reporting a statistically significant increase in enamel Ra after whitening using an acidic 35% HP (pH 6.6) followed by remineralisation using CPP-ACP (Gama Cunha *et al.*, 2012; Abe *et al.*, 2016). Studies considering the impact of CPP-ACP in restoring/preventing changes in whitened enamel roughness, however, show inconsistent results possibly caused by differences in the application duration and frequency. CPP-ACP was reported to significantly reduce enamel Ra after

whitening (Mukarromah *et al.*, 2018). It is worth noting, however, that the remineralising agent was applied for 10 minutes, twice a day, for a duration of 15 days which is a significantly longer application duration and frequency in comparison to the protocol adopted in this current study. Greater application frequency of CPP-ACP on demineralised enamel significantly reduces the damaging effects of demineralising agents (Carvalho *et al.*, 2014).

Enamel roughness significantly increased after whitening using pH9 HP. Furthermore, roughness values were significantly greater in samples whitened using pH9 HP and remineralised using CPP-ACP in comparison to enamel whitened with pH9 HP. CPP-ACP is considered an alkaline salt (pH 7.8) (Khoroushi *et al.*, 2015), therefore combining alkaline salts with an alkaline HP could possibly intensify the damaging effect of the whitening agent on whitened enamel roughness.

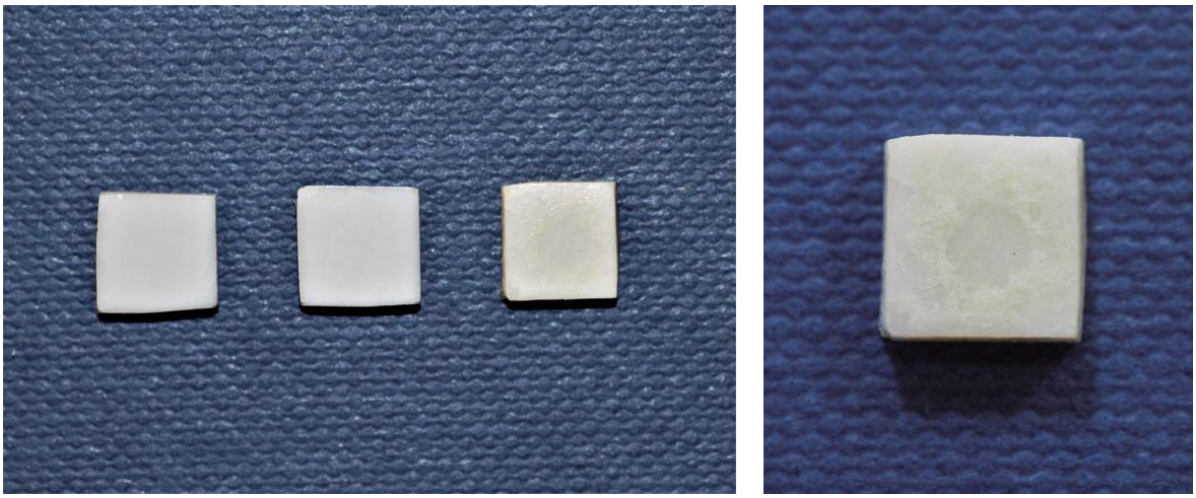


Figure 98 Shows enamel samples whitened using pH5 6%HP then not remineralised (left), remineralised using CPP-ACP (middle), and remineralised using nHa(right). Note the irregular layer of nano crystals formed on enamel treated with nHa in the close-up image.

6.7.2 Enamel Vickers hardness (HV)

As a consequence of dental whitening, enamel hardness reduction is expected (Magalhães *et al.*, 2012). This is caused by the loss of enamel apatite, rendering the surface porous and weaker in comparison to its original state prior to whitening. Applying remineralising agents have shown contradictory results which ignited a controversy around their clinical significance in restoring whitened enamel (Davari *et al.*, 2012; da Costa Soares *et al.*, 2013; Lee *et al.*, 2016b). The application of CPP-ACP in this study did not have a significant remineralising effect on whitened enamel. Hardness reductions recorded did not significantly differ from those recorded in the control enamel samples. This is in agreement with a study by Kutuk *et al.*, revealing no significant differences in enamel hardness reductions after whitening using 38% HP followed by the application of CPP-ACP in comparison to samples whitened with no remineralising agent applied (Kutuk *et al.*, 2019). Furthermore, hardness values of enamel whitened using a 1:2 ratio of 7.5% HP and CPP-ACP did not statistically differ from baseline values after 14 days of treatment according to another study (Vasconcelos *et al.*, 2012). To date, contradictory results on the effectiveness of CPP-ACP in restoring whitened enamel hardness still exist in published literature, some claim that it significantly increases HV values in whitened enamel in comparison to baseline values (Heshmat *et al.*, 2016), while others report its inability to restore enamel hardness to pre whitened conditions (Gama Cunha *et al.*, 2012). Although CPP-ACP stabilises enamel calcium and phosphate ions by maintaining a supersaturated mineral environment (Borges *et al.*, 2011a), differences in the application duration and frequency might explain the inconsistent behaviour of whitened enamel after remineralisation.

Current results additionally showed a significant reduction in enamel Vickers hardness despite the application of nHA after whitening using pH7 and pH9 HP. Enamel treated with pH5 HP, on the other hand, showed no statistically significant reductions in enamel HV after treatment with nHA. This is in agreement with published literature, revealing that hardness values in enamel whitened using HP with a pH value of 5.1 then treated with a nHA remineralising paste did not significantly differ from baseline values (Gomes *et al.*, 2017). This could be attributed to the demineralising solution pH, as it has been reported that the remineralising effect of nHA agents increase significantly at low pH values (Huang *et al.*, 2009; Huang *et al.*, 2011). In contrast, nHA did not restore hardness in enamel whitened using 35% HP with an average pH value of 8.5, revealing significant reductions in enamel hardness post treatment, similar to current results (da Costa Soares *et al.*, 2013). Controversy in reported results could be attributed to the whitening agent pH or the use of commercially available nHA pastes which also contain fluoride and other remineralising additives, which might influence the remineralisation process (da Costa Soares *et al.*, 2013; Loguercio *et al.*, 2015). As previously mentioned in section 6.7.1,

the low solubility of nHA lead to the aggregation of nano-particles and the formation of micro-clusters which block surface pores and restrict access to deeper demineralised enamel (Huang *et al.*, 2009; Swarup and Rao, 2012). Indeed, the use of 15.5% nHA in the current study contributed to the dramatic decrease in enamel hardness values in comparison to other treatment groups as the indenter was probably measuring the hardness of the nHA layer rather than the treated enamel surface.

6.7.3 Enamel colour

Remineralising agents are applied to minimise enamel damage caused by the whitening process without limiting the efficacy of the whitening product. Treatment with CPP-ACP and nHA did not significantly affect the resultant colour of enamel samples whitened using pH7 and pH9 HP as they have produced similar ΔE values as their respective non-remineralised control groups. Indeed, it has been previously reported that nHA and CPP-ACP applied during or after the whitening cycle have shown no significant impact on the whitening effect of HP (de Vasconcelos *et al.*, 2012; Sasaki *et al.*, 2015; Kutuk *et al.*, 2018). Applying CPP-ACP, however, on enamel whitened using pH5 HP resulted in a statistically greater increase in L* values which contributed to a significantly greater ΔE in comparison to samples remineralised using nHA and control enamel samples. According to a study conducted by Shirani *et al*., treatment with CPP-ACP caused a significant increase in L* values in enamel whitened using an acidic 20% CP (Shirani *et al.*, 2015). The application of CPP-ACP was reported to effectively restore rough demineralised enamel, by this enhancing its translucency and lustre (Manton *et al.*, 2008). Therefore, treatment with an acidic HP; reported to produce a frosted glass effect which gives an illusion of a lighter surface (Xu *et al.*, 2011) in combination with CPP-ACP claimed to give enamel a "lighter than normal appearance" could explain the significantly lighter surface in enamel whitened using pH5 HP and remineralised using CPP-ACP in comparison to other treatment groups (Shirani *et al.*, 2015).

Enamel whitened using pH5 and pH9 HP and remineralised using CPP-ACP and nHA showed no significant differences in a* values between treatment groups and in comparison to their respective non-remineralised control groups; similar to previously published research (Borges *et al.*, 2011a; Gomes *et al.*, 2017). In addition, samples treated using pH7 HP showed a significant reduction in a* values only in enamel remineralised using CPP-ACP, however, it was not significantly different than a* values recorded in the control group. This is in line with published research reporting a significant decrease in enamel a* values after whitening using 10% CP (pH 6.5-7) followed by treatment with CPP-ACP (Kim *et al.*, 2011). Furthermore, enamel whitened using 10% and 16% CP then treated with CPP-ACP exhibited significant changes in a* values similar to those recorded in their respective non-remineralised control groups (Borges *et al.*, 2011a). Changes in a* values along the green/red spectrum, however, were reported to have the least effect on the resultant colour in comparison to other colour parameters (Gomes *et al.*, 2017).

Subjective evaluation of colour change following a whitening procedure is strongly associated with changes in (b*) values from yellow to blue than with changes in L* and a* values (Gerlach *et al.*, 2002). Current results showed that enamel b* values significantly decreased after

whitening using pH5, pH7, and pH9 6% HP and remineralisation using CPP-ACP and nHA, which is in line with previously published research (Borges *et al.*, 2011a; de Vasconcelos *et al.*, 2012; Sasaki *et al.*, 2015; Kutuk *et al.*, 2018). Reductions in b* values away from the yellow and towards the blue spectrum were not significantly different between enamel remineralised with CPP-ACP and control enamel samples. On the other hand, the smallest change in b* values occurred in enamel treated with nHA, with values significantly greater than b* values recorded in other treatment groups; indicating a more yellow surface. This could be attributed to the significant increase in enamel roughness caused by the application of nHA as explained in section 6.7.1, creating an irregular enamel surface more susceptible to stain uptake and retention (Shannon *et al.*, 1993; Pinto *et al.*, 2004; Tredwin *et al.*, 2006; Huang *et al.*, 2009; Eva *et al.*, 2013; Hassan *et al.*, 2016). Our results are partially in contrast with results from an *in-situ* study, as whitening using 35% HP then remineralisation using nHA were reported to cause significant changes in all colour parameters similar to those recorded in control enamel samples whitened using 35% HP with no remineralising agent applied (Gomes *et al.*, 2017). Similar changes in b* values between treatment groups could be explained by the treatment duration as the nHA agent was applied for 10 minutes, weekly, for three weeks which is significantly different than the application protocol adopted in this study.

6.7.4 Enamel mineral composition

The purpose of applying remineralising agents during the whitening regime is to restore enamel apatite to its original baseline level (Bayrak *et al.*, 2009). In this study no significant differences were observed in the atomic % of Ca, P, and C between samples whitened using 6% HP with pHs 5,7, and 9 and remineralised using CPP-ACP and nHA in comparison to their respective control groups. Based on current results, whitening using 6% HP did not cause a significant change in enamel mineral content, and the application of remineralising agents did not significantly affect enamel Ca, P, and C levels. Controversy exists around the impact of whitening and remineralisation on enamel mineral composition as some studies have reported no significant differences in mineral content between whitened enamel samples and enamel whitened and remineralised (Coceska *et al.*, 2016; Moreira *et al.*, 2017), while others recorded a significant mineral gain after the application of remineralising agents (Gjorgievska and Nicholson, 2010; Swarup and Rao, 2012; Sajjan *et al.*, 2016; Memarpour *et al.*, 2019). Differences could be attributed to the treatment duration (Sajjan *et al.*, 2016), demineralising enamel with the purpose of creating early enamel lesions; resulting in significant reductions in enamel mineral content, before applying the remineralising agent (Gjorgievska and Nicholson, 2010; Swarup and Rao, 2012; Memarpour *et al.*, 2019), or the destructive preparation process of enamel samples for SEM/EDX; making comparisons between readings from the same enamel sample unachievable (Moreira *et al.*, 2017). According to Coceska *et al.*, samples whitened using 40% HP showed no significant changes in Ca and P according to EDX measurements and enamel whitened and remineralised using CPP-ACP or 30% nHA were reported to have similar Ca and P levels to those recorded in whitened enamel samples (Coceska *et al.*, 2016). Therefore, in the absence of significant changes in enamel mineral content after whitening, it would be reasonable to assume that the remineralisation capacity of the applied CPP-ACP and nHA could not be measured using EDX, similar to current results and results reported in section 5.7.4.

6.7.5 Enamel morphology

Qualitative analysis of enamel microstructure revealed significant surface irregularities in all treatment groups that were remineralised with nHA. This was caused by the aggregation of apatite crystals according to EDX results and accumulation of nanoparticles forming fragile thin overlapping layers of the remineralising agent. The nHA crystals aggregate into microclusters which form an apatite layer on the demineralised enamel surface (Swarup and Rao, 2012), forming a homogeneous apatite coating which cover the prismatic and inter-prismatic enamel structures (Roveri *et al.*, 2009).

Enamel treated with pH5 and pH7 HP and PBS (control) and remineralised with CPP-ACP, on the other hand, showed relatively smooth unaffected surfaces, similar to their respective control groups. The absence of significant microstructural changes could be attributed to the low whitening agent concentration used, which has been previously reported to cause no significant morphological changes to enamel surfaces (Lilaj *et al.*, 2019). Whitening using 6% and 6.5% HP caused no significant morphological damage to treated enamel (White *et al.*, 2003; Duschner *et al.*, 2006). In fact, whitening agent concentrations as high as 38% HP were reported to cause no significant changes in enamel microstructure, and the application of remineralising agents such as CPP-ACP had no significant effect on enamel morphology according to SEM (Kutuk *et al.*, 2019).

Current results additionally showed that treatment with pH9 HP caused significant damage to enamel microstructure visible as the loss of prismatic and interprismatic enamel, which is expected from an alkaline solution reported to target organic matter (Taube *et al.*, 2010). The dissolution of enamel organic matter and the subsequent loss of the protein rich matrix surrounding enamel prisms will in turn cause the loss of intact prismatic apatite. Applying CPP-ACP after whitening using pH9 HP, on the other hand, appeared to prevent the expected surface damage and maintained a smooth flat unaffected whitened surface. CPP-ACP has been reported to cover the interprism cavities and enamel prismatic structures, by forming a smooth resistant layer to future attacks (Poggio *et al.*, 2013). Although treating whitened enamel with CPP-ACP was effective in preventing microstructural damage caused by the alkaline HP, this did not however reflect an improved enamel roughness, hardness, colour, or mineral composition in comparison to its respective control.

6.8 Conclusion

Remineralising whitened enamel using nHA significantly reduced HV, increased Ra, resulted in lower overall colour change (ΔE), and significantly damaged enamel microstructure. Enamel whitened using pH7 HP and remineralised using CPP-ACP, on the other hand, showed no significant changes in Ra and HV as compared to baseline values. Additionally, enamel treated with pH5 HP and remineralised using CPP-ACP showed a significant increase in Ra and a significant decrease in HV after treatment, all of which were not significantly different from results recorded in the control group. The application of CPP-ACP caused a significantly rougher enamel in samples whitened using pH9 HP in comparison to control enamel samples. Hardness values, however, did not significantly differ between enamel treated with CPP-ACP and control samples.

Treatment with CPP-ACP and nHA produced similar ΔE values in enamel whitened using pH7 and pH9 HP as their respective non-remineralised control groups. Applying CPP-ACP, however, on enamel whitened using pH5 HP resulted in a significantly greater ΔE in comparison to samples remineralised using nHA and control enamel samples. The combined whitening/ remineralisation protocols did not significantly affect mineral composition and the application of CPP-ACP was effective in preventing microstructural damage only in enamel treated with pH9 HP.

In summary, the use of 15.5% nHA as a remineralising agent did not restore enamel to pre-whitened conditions as the aggregation of apatite crystals on treated enamel has created structurally weak and rough surfaces; more susceptible to stain uptake and discolouration. The use of CPP-ACP on the other hand, significantly improved the resultant colour in samples whitened using pH5 HP, and prevented morphological damage in samples whitened using pH9 HP.

Chapter 7. Phase III: The effects of dietary erosion and staining on whitened/remineralised enamel

7.1 Introduction

Dental enamel is subjected to dietary staining and erosion on a regular basis (West *et al.*, 2000; Mullan, 2018). Therefore, dental whitening studies must consider the longevity of whitening results under these circumstances, and strive to maximise these desired results by attempting to minimise post whitening enamel staining and erosive damage caused by dietary products regularly consumed by the general public.

For that reason, this novel approach in testing and evaluating the effects remineralising agents have on enamel whitened using different HP pH values in resisting erosive damage and stain uptake will be important to help achieve and maintain the desired whitening outcome.

7.2 Study aims

1- Study the vulnerability of bovine enamel to dietary erosion after being whitened using acidic, neutral, and alkaline 6% HP and remineralised using CPP-ACP or nHA in terms of enamel roughness, hardness, mineral composition, and surface quality.

2- Study the vulnerability of bovine enamel to dietary staining after being whitened using acidic, neutral, and alkaline 6% HP and remineralised using CPP-ACP or nHA based on enamel colour measurements.

7.3 Materials and methods

After completing phase 2 as described in chapter 6, enamel whitened (n=36 per HP pH) and remineralised with nHA (n=12) or CPP-ACP (n=12), and control samples (n=12) were divided into two groups; the first group (n=5) was subjected to simulated dietary erosion, the second group (n=5) was subjected to simulated dietary staining, and the remaining two samples were set aside for SEM/EDX measurements.

7.3.1 Simulated dietary erosion

Enamel samples were immersed in a 0.3% citric acid solution for 15-minutes, daily, for three consecutive days. The citric acid solution was made by dissolving 0.3g of citric acid (Fisher Scientific©, Leicestershire, UK) in 100mL distilled water. The solution pH was then modified, using 0.5M NaOH, to a pH of 3.8. The erosion cycle was performed at room temperature and the solution was agitated at 60 rpm on a digital hot plate magnetic stirrer (VELP, Scientifica, Italy). Roughness and hardness measurements were obtained at baseline and after each erosion cycle using atomic force microscopy (n=4 per group) (NanoWizard® 3 NanoOptics AFM system, JPK Instruments, Berlin, Germany) and a universal test machine (n=5 per group) (Zwick Z 2.5, Zwick GmbH & Co., Ulm, Germany), respectively. After treatment, qualitative evaluation of enamel microstructure (n=2 per group) was undertaken using Tescan Vega 3LMU SEM (Tescan Vega SEM LMU, Tescan, Cambridge) and quantitative assessment of mineral composition was obtained using energy dispersive x-ray spectroscopy (EDX) (Bruker Xflash 6130, Bruker®, Camarillo, CA). Samples were stored in artificial saliva at 37°C between treatments.

7.3.2 Simulated dietary staining

Samples were immersed in coffee for three consecutive five-minute cycles, daily, for three consecutive days. Coffee was made by diluting 1.8g of a commercially available instant coffee (Nescafe© Original, Nestle, York, UK) in 200mL of boiling tap water using an electric kettle. The beverage was then allowed to cool down to 60°C. The staining cycle was performed on a digital hot plate magnetic stirrer (VELP, Scientifica, Italy) at 60°C and the solution was agitated at 60 rpm. Colour measurements were obtained for all samples using a spectrophotometer (Ci62, X-Rite Europe GmbH, Regensdorf, Switzerland) at baseline and after each staining cycle, and samples were stored in artificial saliva at 37°C between treatments.

7.4 Study design

The programme of work is shown in Figure 99

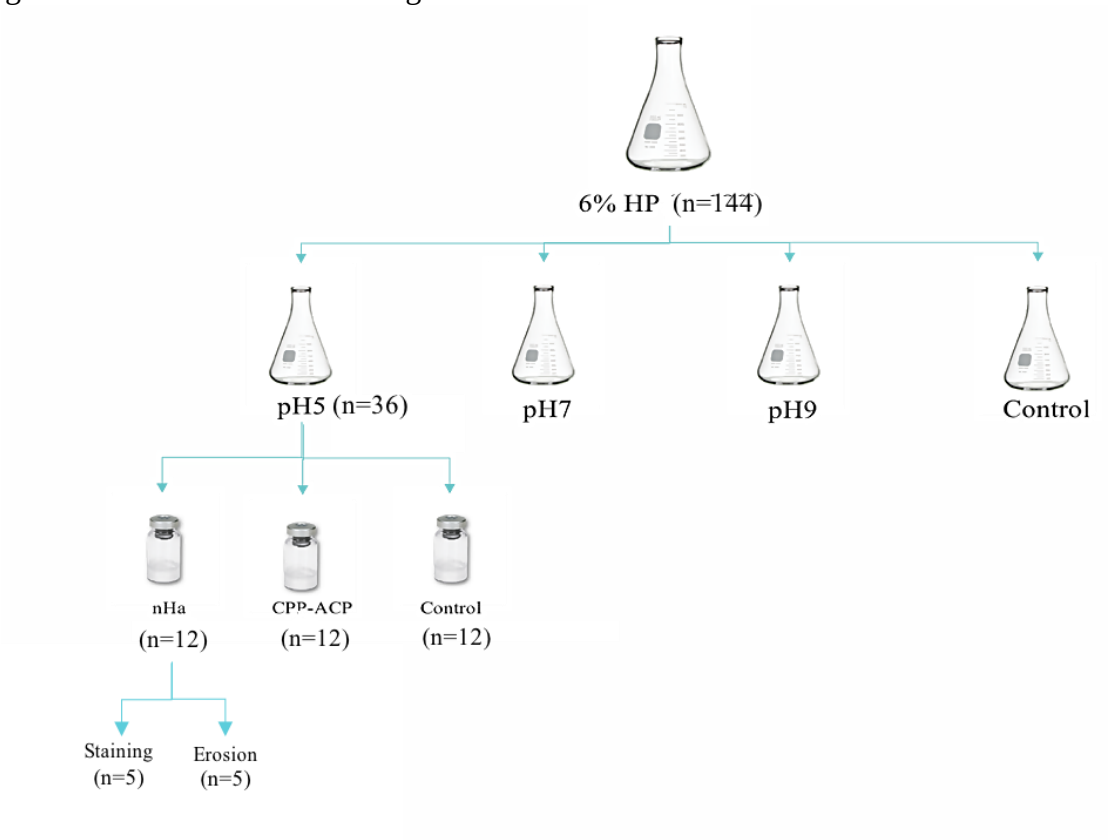


Figure 99 An illustration of the study design. Phase 3 was conducted by dividing the nHA, CPP-ACP, and control groups treated with pH5, pH7, pH9 HP, and PBS in Phase2 into two subgroups each, the first exposed to a dietary acid and the second exposed to a dietary stain.

7.5 Statistical analysis

Statistical analysis was performed using Sigma Plot© for Windows version 13.0 build 13.0.0.83 (Systat Software 2014®). Statistical analysis of data was performed using Kruskal-Wallis One Way Analysis of Variance on Ranks for non-parametric data, while normally distributed data were analysed using One Way Analysis of Variance. Normality tests were performed using Shapiro-Wilk, and equal variance was tested using Brown-Forsythe. Pairwise multiple comparisons were done using Tukey test with an overall significance of 0.05.

7.6 Simulated dietary erosion results

7.6.1 Enamel whitened using pH5 Hydrogen Peroxide

Enamel surface topography

Surface topography images revealed a gradual exposure of enamel prisms and consequent increase in surface irregularities in the control group after each erosion cycle (Figure 100). The CPP-ACP group, on the other hand, showed a smooth flat unaffected enamel surface after the first erosion cycle, followed by a highly irregular surface as a result of the second erosion cycle appearing as sharp peaks which turned blunt after the third erosion cycle. Eroding the nHA group caused a rougher surface after the second and third erosion cycles.

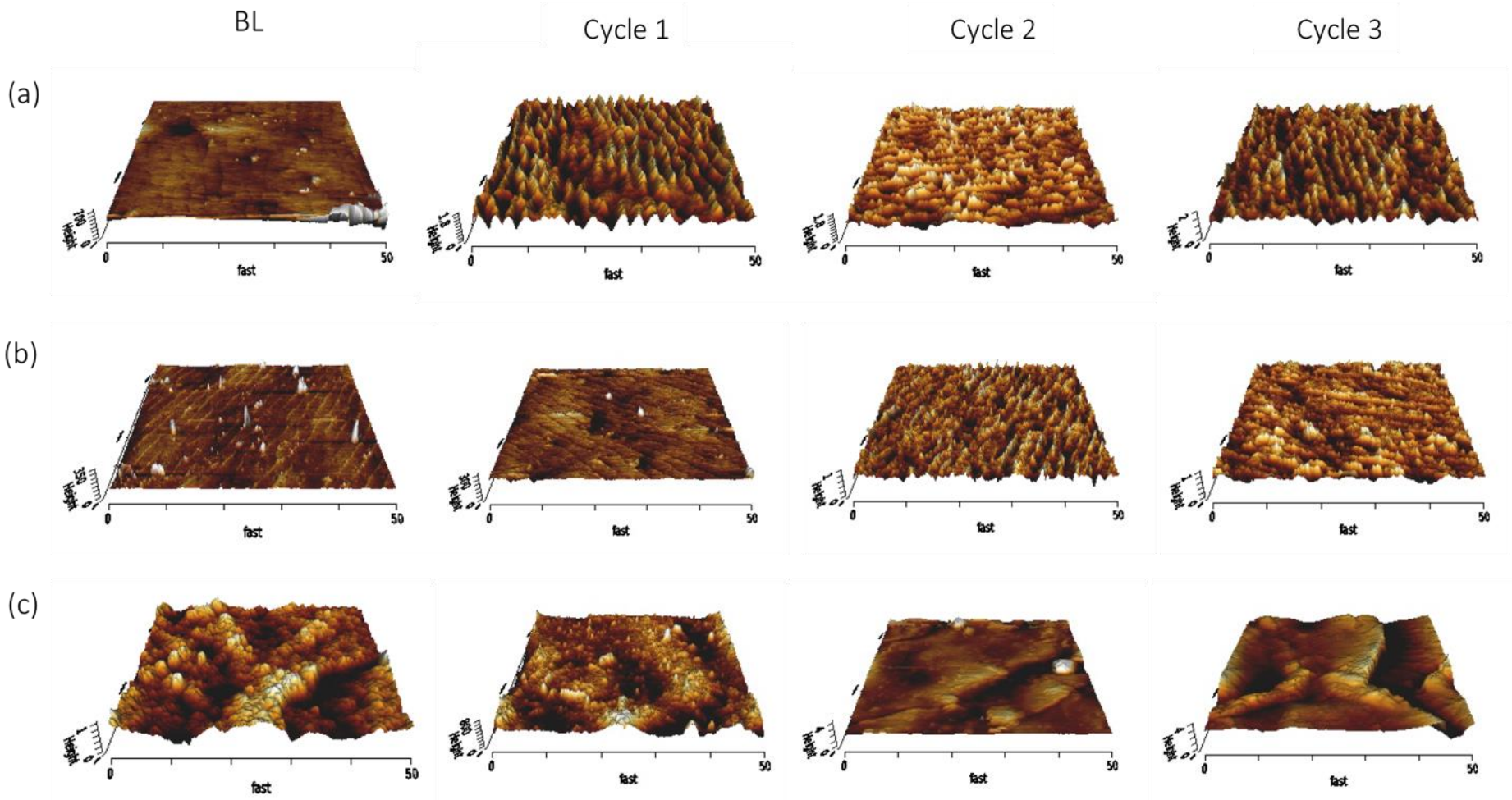


Figure 100 Representative AFM 3D images of whitened bovine enamel not remineralised (a), remineralised with CPP-ACP (b), and remineralised with nHA (c) at BL, Cycle 1, Cycle 2, and Cycle 3 of erosion. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-axis is in µm in all images captured except in group (a) and (b) at BL and group (b) and (c) after cycle 1 which are in nm.

Enamel roughness (Ra)

A significant increase in enamel Ra was recorded in the control group after all erosion cycles in comparison to BL ($P \leq 0.05$). Enamel remineralised using CPP-ACP showed no significant change in Ra after the first erosion cycle ($P > 0.05$), then a significant roughness increase was noted after the second and third erosion cycles ($P \leq 0.05$). The nHA group on the other hand showed no significant changes in roughness values after each erosion cycle, with great variability in Ra values recoded ($P > 0.05$) (Table 55) (Figure 101).

	Ra (nm)			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	7.2 (3.1)[a]	112.7 (102.1)[b]	141.9 (53.6)[b]	132.7 (54.7)[c]
CPP-ACP	6.7 (4.3)[a]	7.1 (65.2)[a]	98.6 (85.8)[b]	87.8 (86.6)[b]
nHA	127.3 (65.4)[a]	101.5 (76.1)[a]	121.2 (107.3)[a]	124.9 (115.0)[a]

Table 55 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH5 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

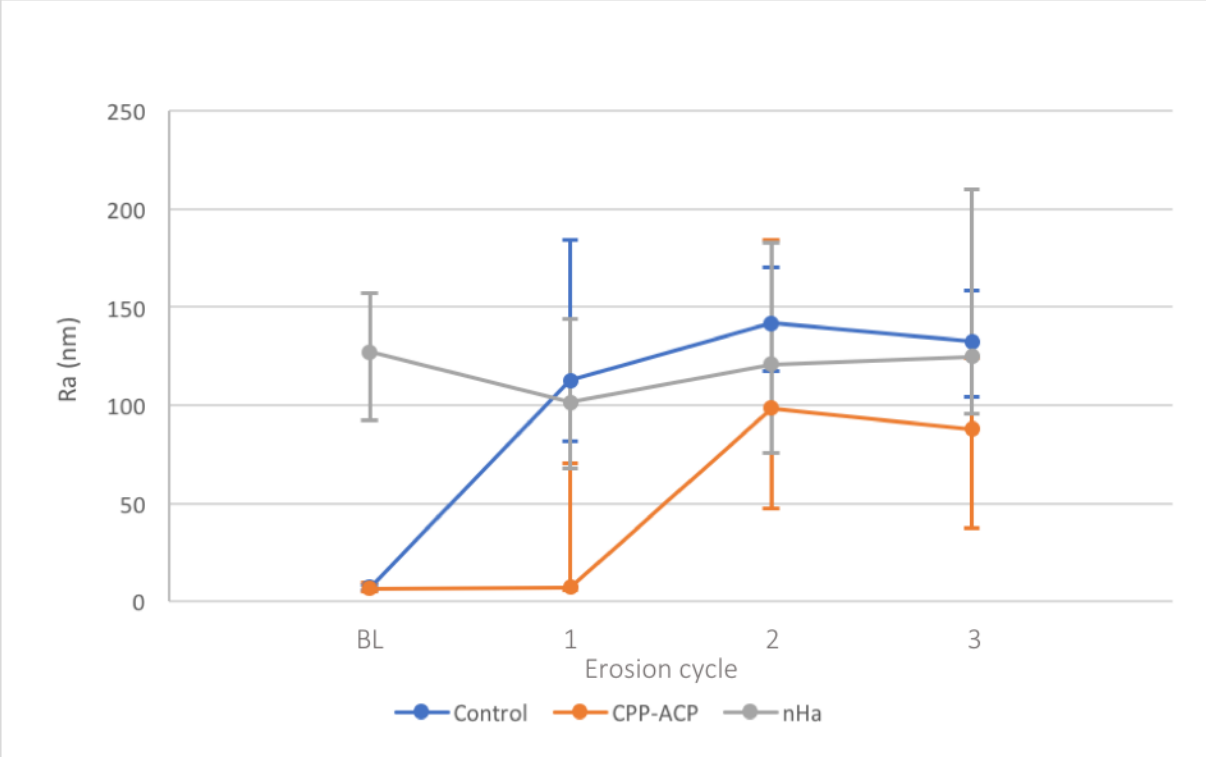


Figure 101 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH5 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Enamel hardness (HV)

Control enamel samples showed a significant decrease in HV after the first, second, and third erosion cycles in comparison to BL ($P \leq 0.05$). Enamel hardness reductions, however, were not significantly different after the second and third erosion cycles ($P > 0.05$). Similarly, enamel treated with CPP-ACP showed a significant decrease in HV after the first and third erosion cycles in comparison to BL ($P \leq 0.05$). Enamel HV reductions were not significantly different after the second and third erosion cycles in comparison to hardness values obtained after the first erosion cycle ($P > 0.05$). Enamel treated with nHA showed no significant changes in hardness values after each erosion cycle, with great variability in HV values recoded ($P > 0.05$) (Table 56) (Figure 102).

	HV (H_{IT})			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	333.7 (35.6)[a]	262.0 (59.3)[b]	264.6 (40.4)[b]	245.5 (107.0)[b]
CPP-ACP	288.8 (41.2)[a]	215.8 (60.5)[b]	231.3 (79.3)[a,b]	213.8 (121.7)[b]
nHA	272.1 (124.9)[a]	154.5 (100.5)[a]	189.9 (131.7)[a]	218.0 (105.7)[a]

Table 56 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH5 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

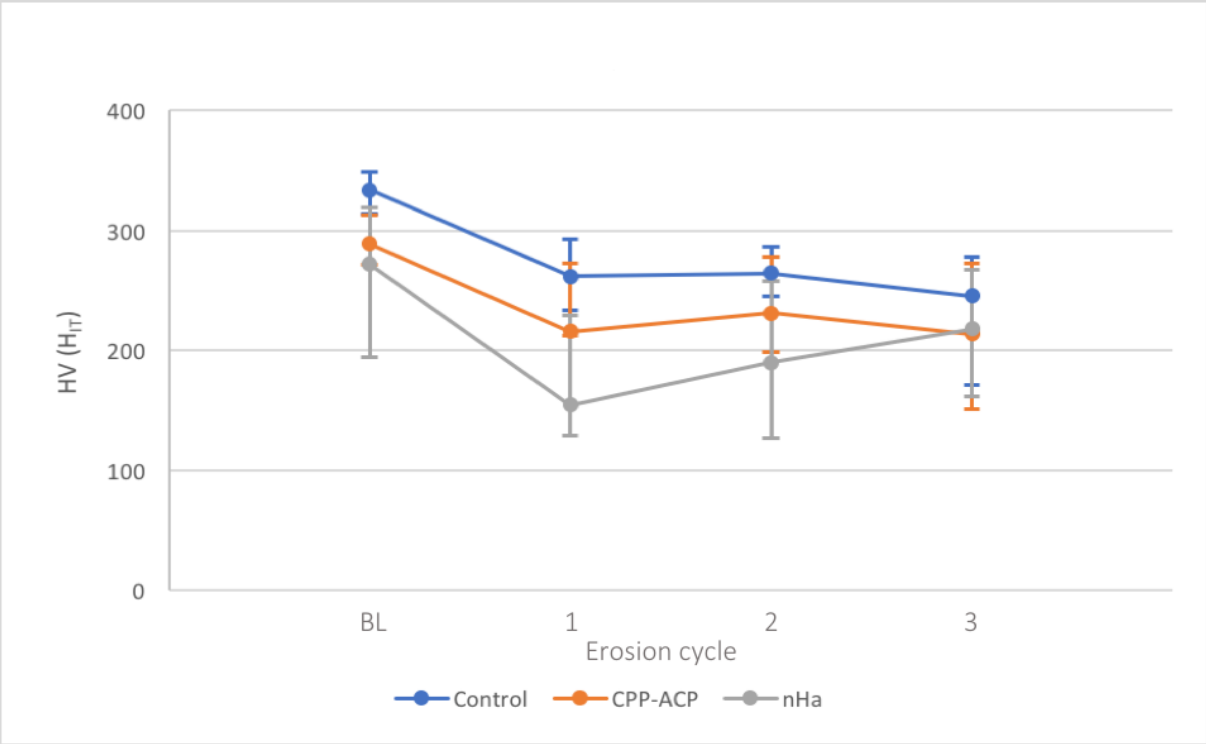


Figure 102 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH5 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Mineral composition

After exposure to three erosion cycles, atomic % of enamel calcium, phosphorus, and carbon were not significantly different between treatment groups (P>0.05) (Table 57).

Group-top	Element	Atomic %
nHA	Ca	23.2 (0.42)
	P	20.2 (1.3)
	C	14.7 (1.9)
CPP-ACP	Ca	24.3 (0.4)
	P	27.4 (7.6)
	C	21.8 (12.8)
Control	Ca	20.6 (5.7)
	P	22.2 (2.8)
	C	25.2 (17.1)

Table 57 Mean atomic % and standard deviation (SD) of eroded enamel calcium, phosphorous, and carbon after whitening using pH5 HP and remineralisation using CPP-ACP or nHA in comparison to a control group.

Surface morphology

After erosion enamel remineralised using nHA exhibited a rough, irregular, and demineralised surface with visible patches of nHA crystals partially covering the treated enamel. Control enamel and samples treated with CPP-ACP were additionally demineralised after dietary erosion; visible as surface irregularities, pitting, and the exposure of enamel prisms (Figure 103-105).

nHA

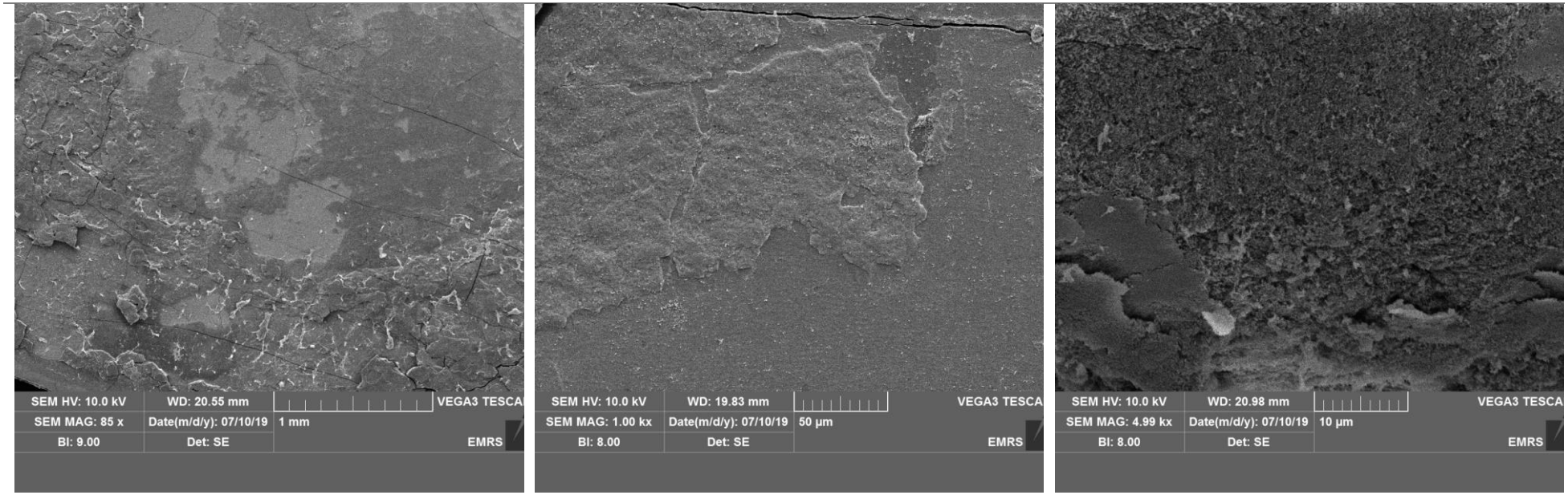


Figure 103 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP and remineralised using 15.5% nHA, then subjected to three erosion cycles. Note enamel surface deformation in addition to the accumulation of nHA crystals.

CPP-ACP

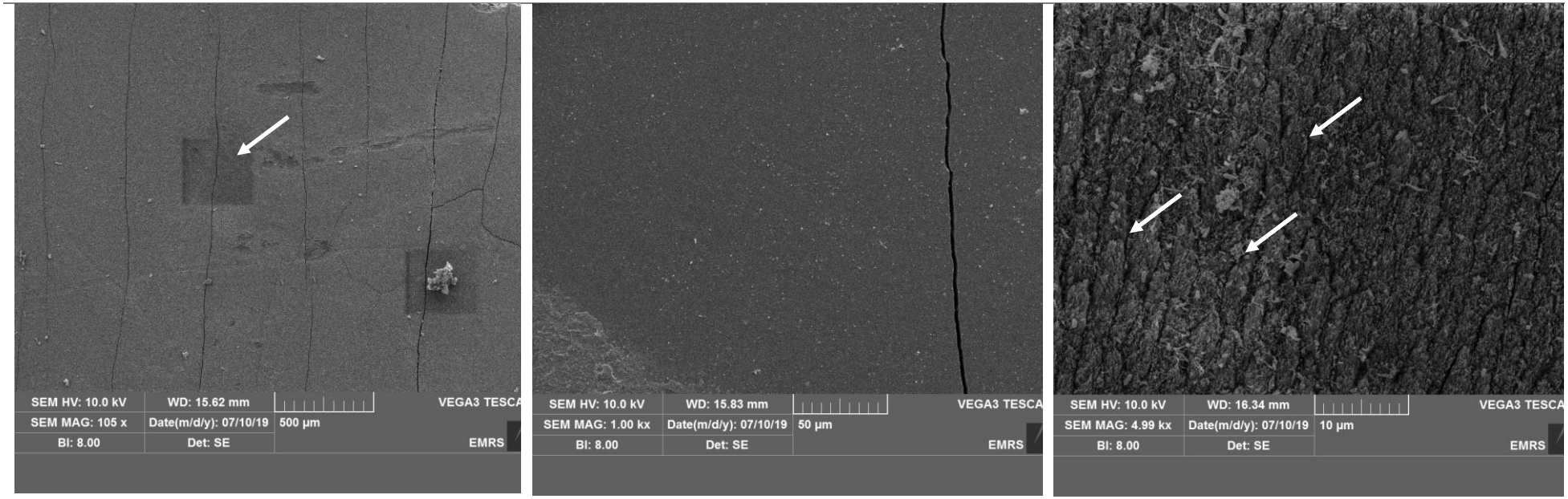


Figure 104 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP and remineralised using CPP-ACP, then subjected to three erosion cycles. Note the exposed enamel prisms under high magnification (arrow), in addition to marks left by the diamond hardness indenter under low magnification (arrow).

Control

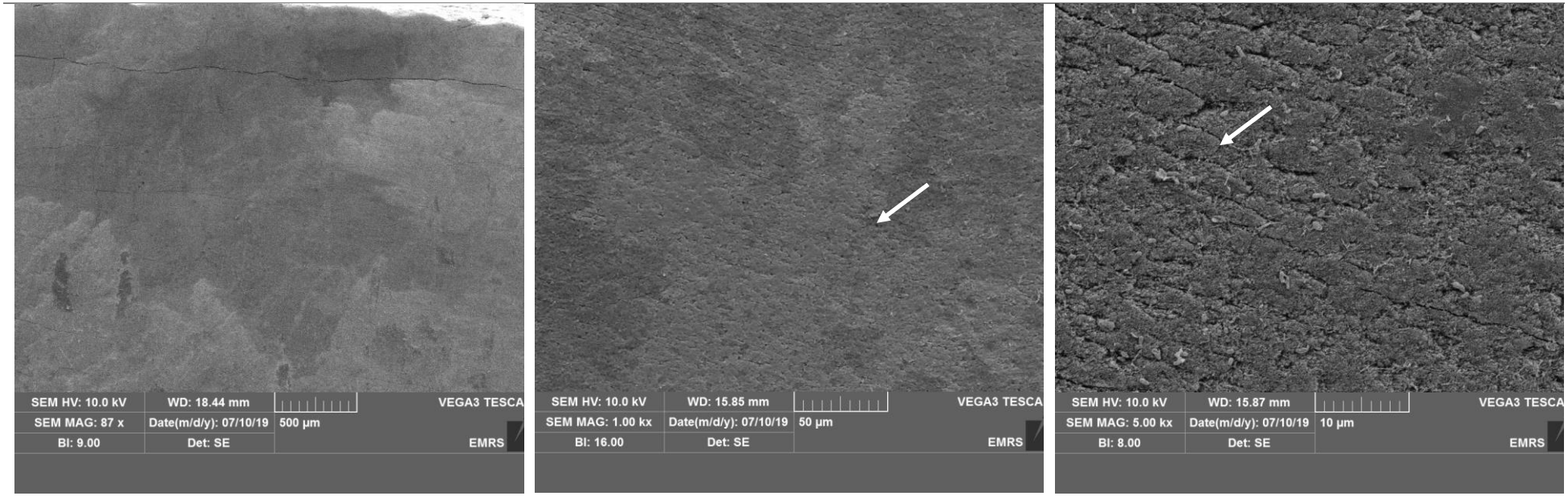


Figure 105 Three SEM images under low, medium, and high magnification of enamel samples treated with pH5 6% HP then subjected to three erosion cycles. Changes were noted in treated enamel under all magnifications. Demineralisation was noted under low magnification, pits under medium magnification (arrow), and exposed enamel prisms visibly seen under high magnification (arrow).

7.6.2 Enamel whitened using pH7 Hydrogen Peroxide

Enamel surface topography

Surface topography images revealed a gradual exposure of enamel prisms and consequent increase in surface irregularities in the control group after all erosion cycles (Figure 106). The CPP-ACP group, on the other hand, showed a smooth flat unaffected enamel surface after the first erosion cycle, followed by a highly irregular surface as a result of the second erosion cycle appearing as sharp peaks which turned blunt after the third erosion cycle. Enamel treated with nHA maintained an irregular surface after all erosion cycles.

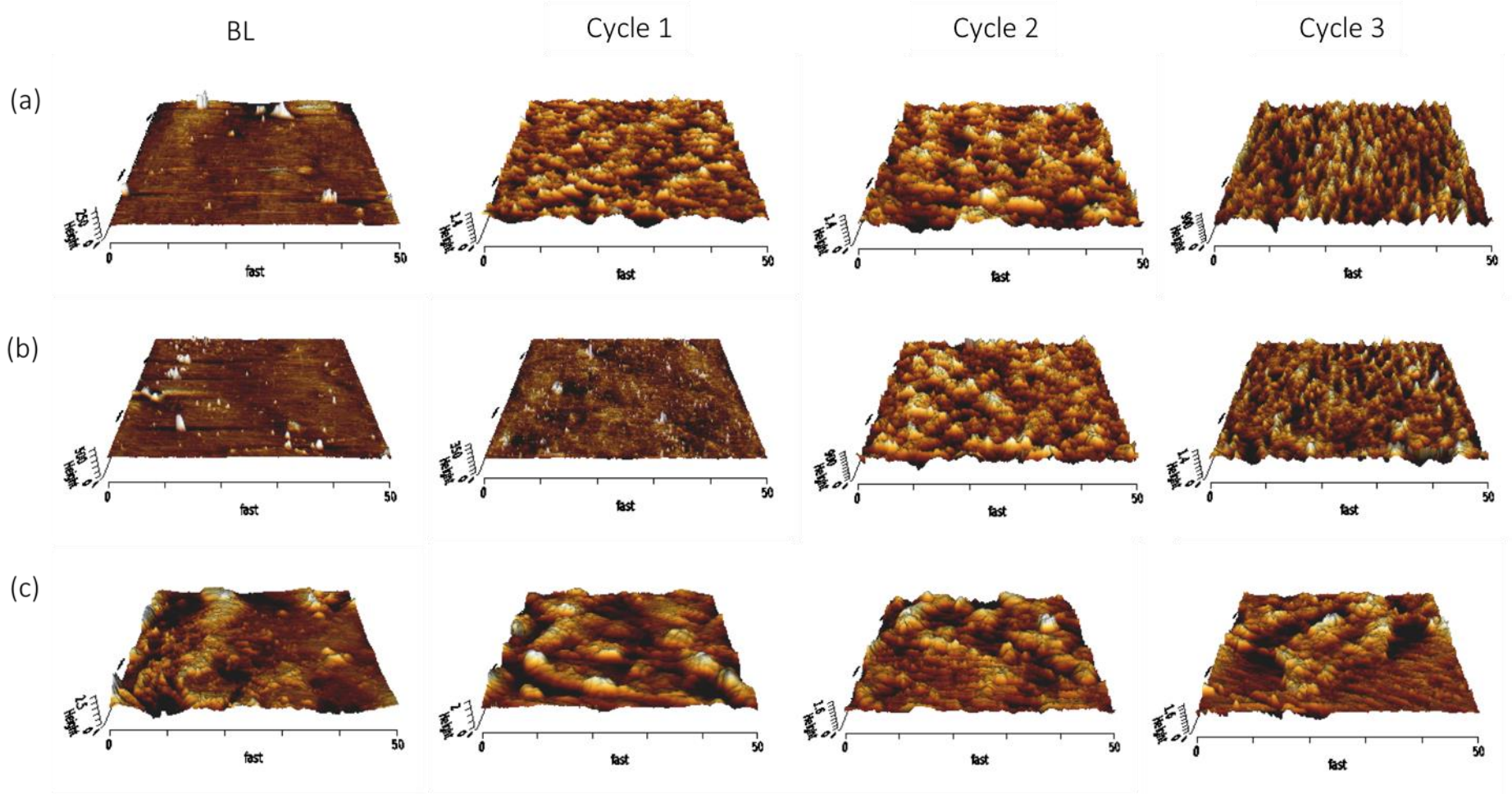


Figure 106 Representative AFM 3D images of whitened bovine enamel not remineralised (a), remineralised with CPP-ACP (b), and remineralised with nHA (c) at BL, Cycle 1, Cycle 2, and Cycle 3 of erosion. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-axis is in µm in all images captured except in group (a) at BL and after cycle 3 and group (b) at BL and after cycle 1 and cycle 2 which are in nm.

Enamel roughness (Ra)

Control enamel samples showed a significant increase in Ra values after each erosion cycle ($P \leq 0.05$). Enamel remineralised using CPP-ACP showed no significant change in Ra after the first erosion cycle ($P > 0.05$), then a significant roughness increase was noted after the second and third erosion cycles ($P \leq 0.05$). Samples treated with nHA showed no significant changes in Ra values after the first and second erosion cycles ($P > 0.05$) and a significant reduction in roughness values were recorded after the third erosion cycle ($P \leq 0.05$). The greatest variability in Ra values were recorded in enamel treated with nHA (Table 58) (Figure 107).

	Ra (nm)			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	7.3 (2.7)^{a}	22.6 (46.3)^{b}	78.8 (55.1)^{c}	112.6 (23.5)^{d}
CPP-ACP	5.3 (3.0)^{a}	15.6 (34.9)^{a}	73.7 (72.8)^{b}	102.9 (66.9)^{c}
nHA	219.9 (299.9)^{a}	245.8 (196.9)^{a}	204.6 (177.2)^{a}	134.8 (112.0)^{b}

Table 58 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH7 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

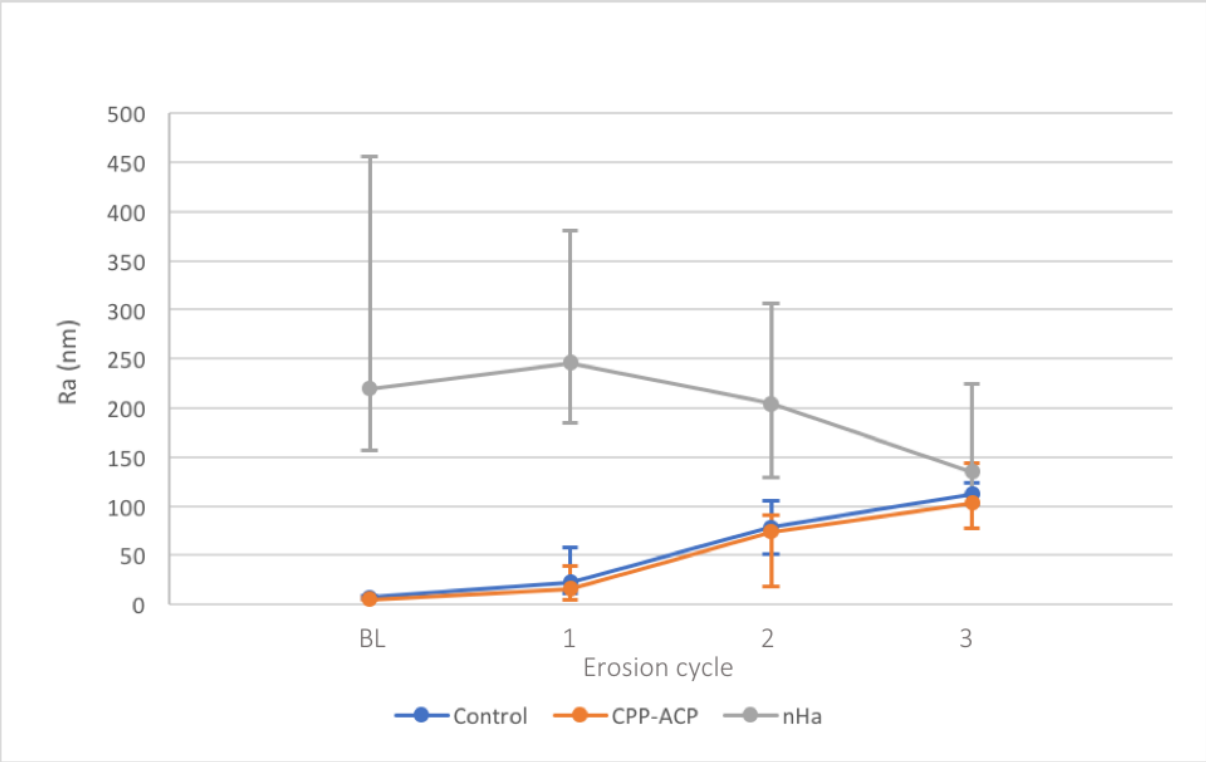


Figure 107 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH7 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Enamel hardness (HV)

Control enamel and samples treated with nHA showed no significant changes in hardness after the first, second, and third erosion cycles ($P>0.05$). Enamel treated with CPP-ACP, on the other hand, showed a significant hardness reduction after the second erosion cycle ($P\leq0.05$), while enamel hardness values after the first and third erosion cycles did not significantly differ from BL or from values recorded after the second erosion cycle ($P>0.05$) (Table 59) (Figure 108).

	HV (H_{IT})			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	269.7 (35.0)[a]	268.1 (78.7)[a]	270.2 (95.9)[a]	240.5 (35.3)[a]
CPP-ACP	299.2 (51.6)[a]	282.2 (83.3)[a,b]	241.6 (35.8)[b]	261.6 (70.7)[a,b]
nHA	206.2 (179.5)[a]	246.6 (213.0)[a]	245.1 (173.7)[a]	201.0 (96.2)[a]

Table 59 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH7 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

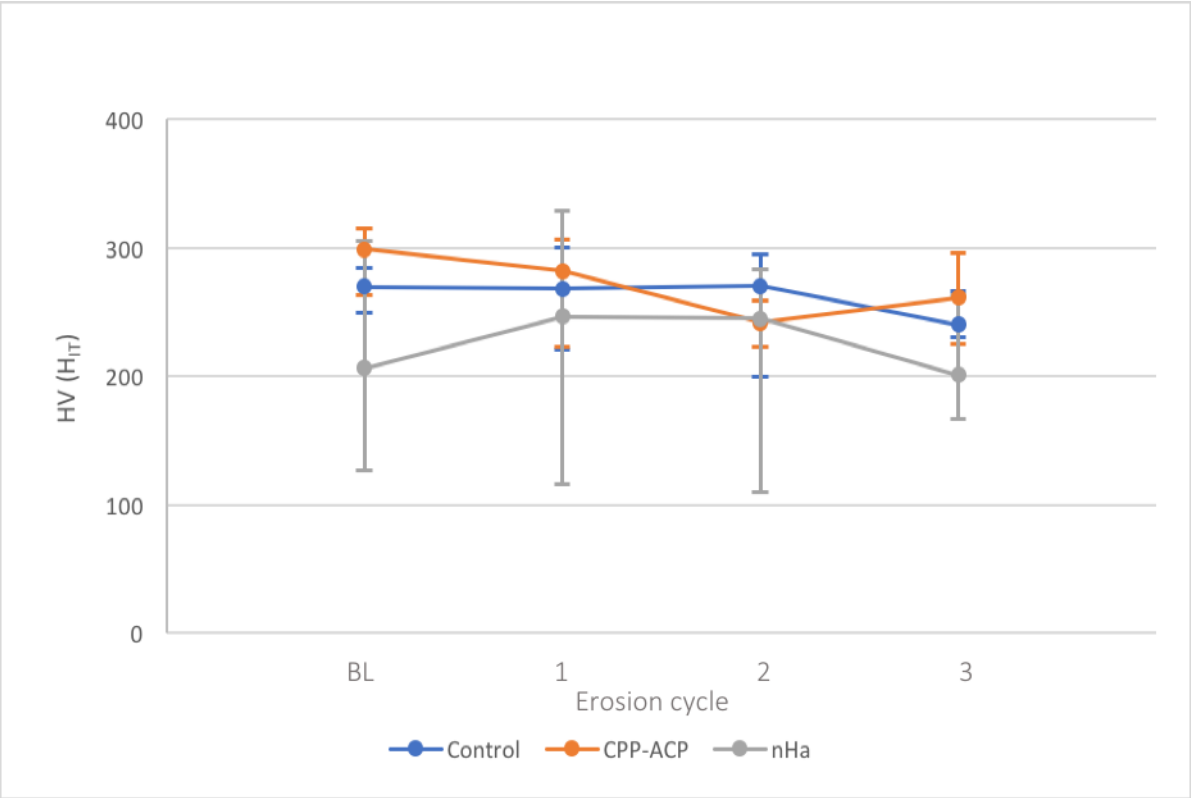


Figure 108 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH7 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Mineral composition

After exposure to three erosion cycles, atomic % of enamel calcium, phosphorus, and carbon were not significantly different between treatment groups (P>0.05) (Table 60).

	Element	Atomic %
nHA	Ca	30.0 (5.4)
	P	21.9 (3.8)
	C	26.2 (2.0)
CPP-ACP	Ca	24.5 (4.9)
	P	21.7 (0.1)
	C	33.1 (8.3)
Control	Ca	24.9 (3.4)
	P	23.0 (1.8)
	C	34.0 (0.8)

Table 60 Mean atomic % and standard deviation (SD) of eroded enamel calcium, phosphorous, and carbon after whitening using pH7 HP and remineralisation using CPP-ACP or nHA in comparison to a control group.

Surface morphology

After erosion enamel remineralised using nHA exhibited a rough, irregular, and demineralised surface with visible patches of nHA crystals partially covering the treated enamel. Control enamel and samples treated with CPP-ACP were additionally demineralised after dietary erosion; visible as surface irregularities, pitting, and the exposure of enamel prisms. In the CPP-ACP group enamel prisms appear to be partially covered with CPP-ACP crystals under high magnification (Figure 109-111).

nHA

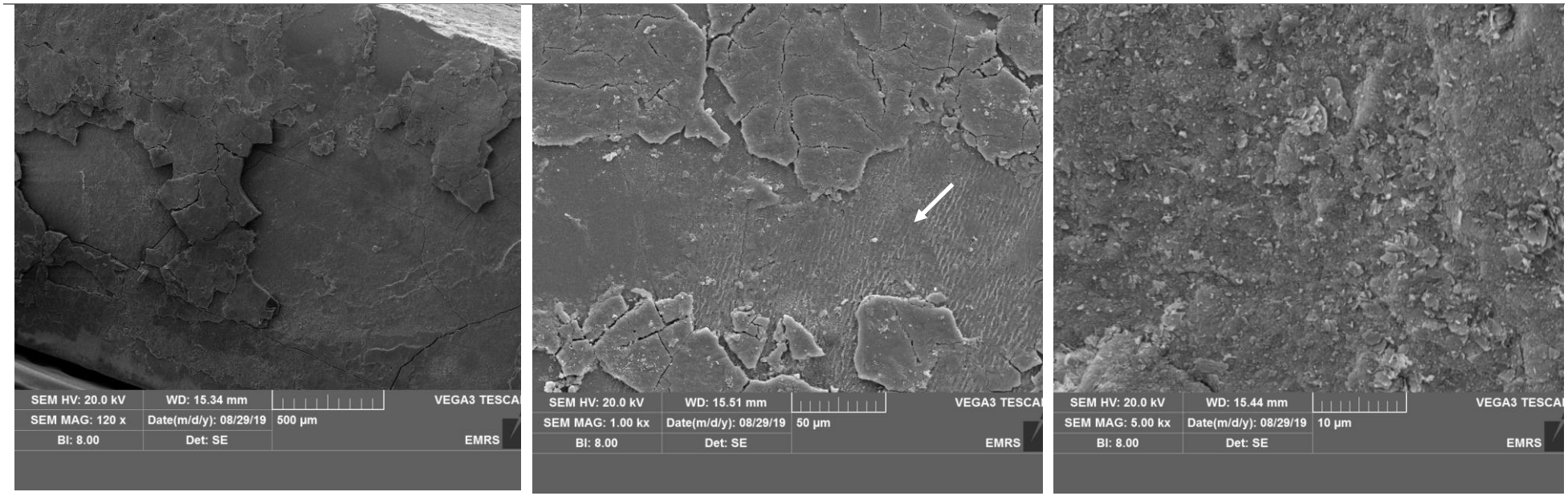


Figure 109 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP and remineralised using 15.5% nHA, then subjected to three erosion cycles. Note the eroded enamel surface (arrow) in addition to the accumulation of nHA crystals.

CPP-ACP

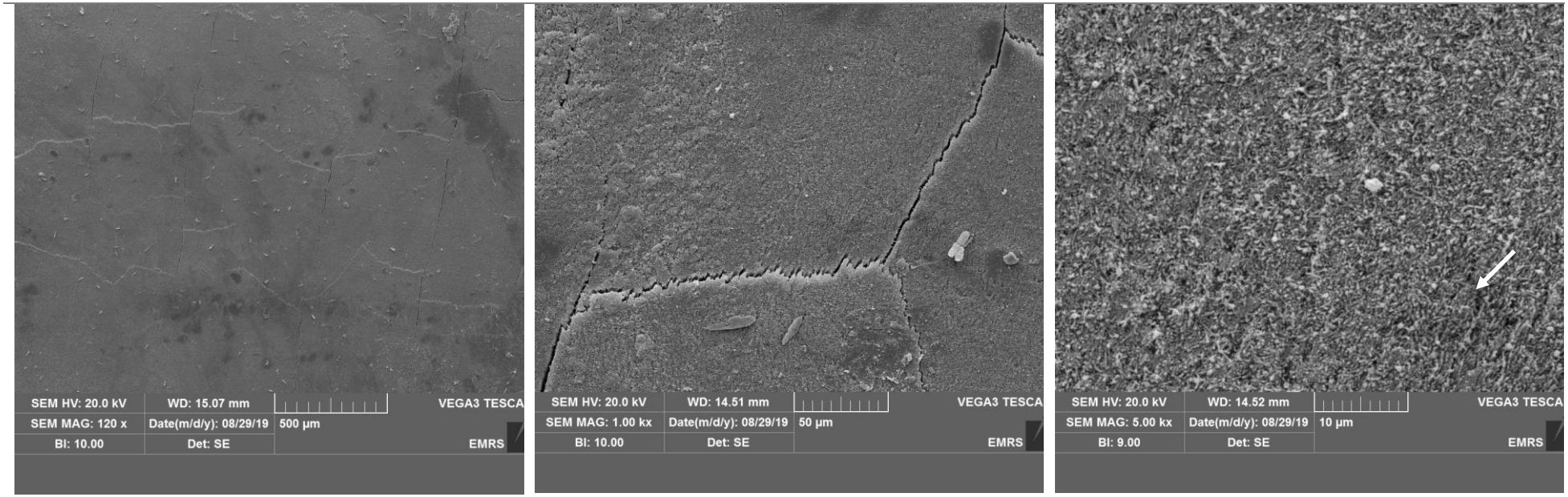


Figure 110 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP and remineralised using CPP-ACP, then subjected to three erosion cycles. Changes were noted in treated enamel under all magnifications. Enamel prisms were exposed under high magnification, however, it appears that enamel prisms were covered with CPP-ACP crystals.

Control

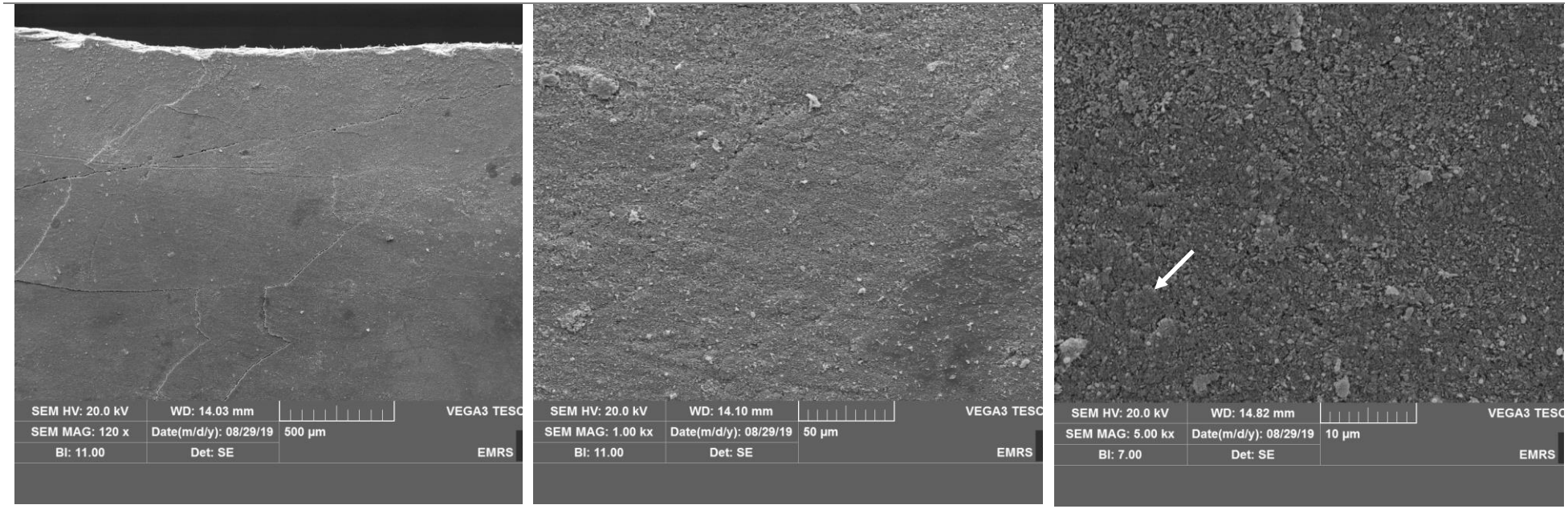


Figure 111 Three SEM images under low, medium, and high magnification of enamel samples treated with pH7 6% HP, then subjected to three erosion cycles. Changes were noted in treated enamel under all magnifications. Enamel prisms were exposed under high magnification (arrow).

7.6.3 Enamel whitened using pH9 Hydrogen Peroxide

Enamel surface topography

Surface topography images revealed a gradual exposure of enamel prisms and consequent increase in surface irregularities in the control group after each erosion cycle. After the first erosion cycle sharp wide peaks were noted, which became more rounded and narrower after the second and third erosion cycles. The CPP-ACP group, on the other hand, showed a smooth flat unaffected enamel surface after the first erosion cycle, followed by a highly irregular surface as a result of the second erosion cycle appearing as sharp peaks which turned blunt after the third erosion cycle. Enamel treated with nHA maintained an irregular surface after all erosion cycles (Figure 112).

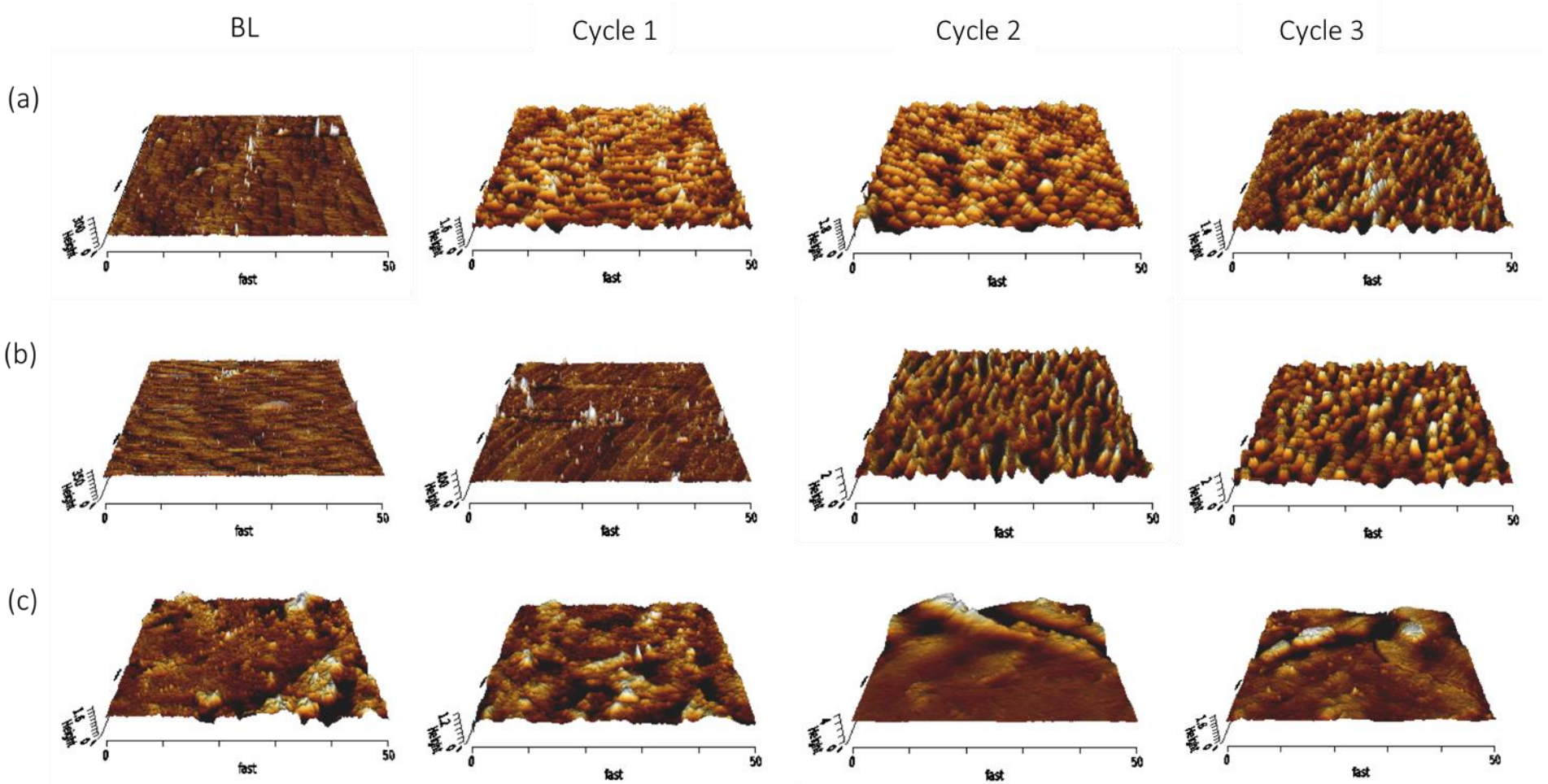


Figure 112 Representative AFM 3D images of whitened bovine enamel not remineralised (a), remineralised with CPP-ACP (b), and remineralised with nHA (c) at BL and after Cycle 1, Cycle 2, and Cycle 3 of erosion. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-axis is in µm in all images captured except in group (a) and (b) at BL and group (b) after cycle 1 which are in nm.

Enamel roughness (Ra)

Control enamel samples showed a significant increase in Ra after the first, second, and third erosion cycles (P≤0.05) (Table 61) (Figure 113). Enamel remineralised using CPP-ACP showed no significant change in Ra after the first erosion cycle (P>0.05) and a significant roughness increase was noted after the second and third erosion cycles (P≤0.05). Samples treated with nHA showed no significant changes in Ra after the first and third erosion cycles (P>0.05) while a significant increase in roughness was recorded after the second erosion cycle (P≤0.05). Roughness values recorded after the second erosion cycle, however, were not significantly different than those recorded after the third erosion cycle (P>0.05).

	Ra (nm)			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	7.7 (3.6)^{a}	9.1 (93.4)^{b}	132.2 (61.1)^{c}	105.3 (138.2)^{c}
CPP-ACP	9.0 (4.1)^{a}	7.3 (86.4)^{a}	86.9 (235.1)^{b}	183.2 (72.5)^{b}
nHA	127.6 (115.4)^{a}	150.7 (182.2)^{a}	208.6 (146.5)^{b}	163.3 (110.2)^{a,b}

Table 61 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH9 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

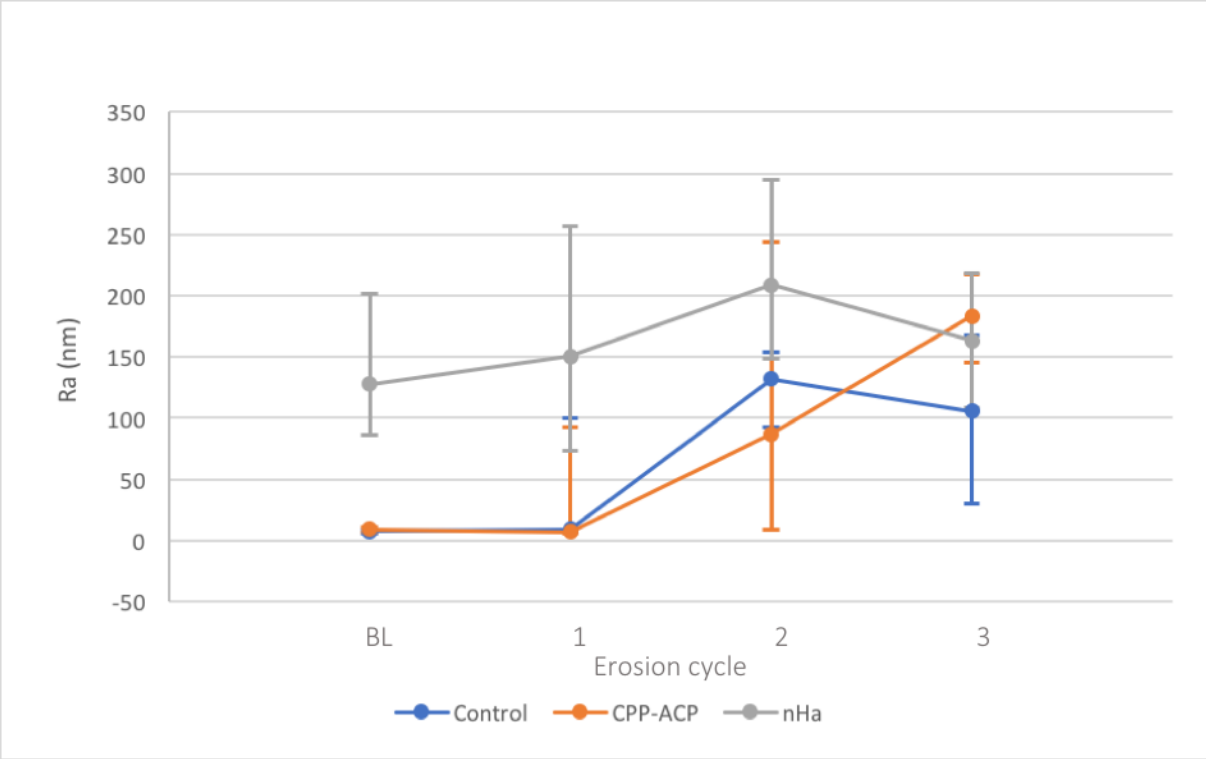


Figure 113 Median (IQR) Ra values of enamel at baseline i.e. after treatment using pH9 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Enamel hardness (HV)

Control enamel samples showed a significant decrease in HV after all erosion cycles in comparison to BL ($P \leq 0.05$) (Table 62) (Figure 114). Hardness values, however, were not significantly different after the first, second, and third erosion cycles ($P > 0.05$). Enamel treated with CPP-ACP showed a significant decrease in HV after the third erosion cycles in comparison to BL ($P \leq 0.05$). After the second erosion cycle, enamel hardness values were similar to those recorded at BL and after the third erosion cycle ($P > 0.05$). Enamel treated with nHA, on the other hand, showed no significant changes in hardness after each erosion cycle ($P > 0.05$), with great variability in HV values recoded.

	HV (H_{IT})			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	287.0 (57.7)[a]	222.1 (108)[b]	201.6 (29.8)[b]	212.5 (65.3)[b]
CPP-ACP	256.2 (56.4)[a]	282.7 (24.4)[b]	230.8 (43.1)[a,c]	200.0 (31.7)[c]
nHA	146.4 (134.8)[a]	126.5 (173.0)[a]	109.8 (130.7)[a]	145.5 (62.7)[a]

Table 62 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH9 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

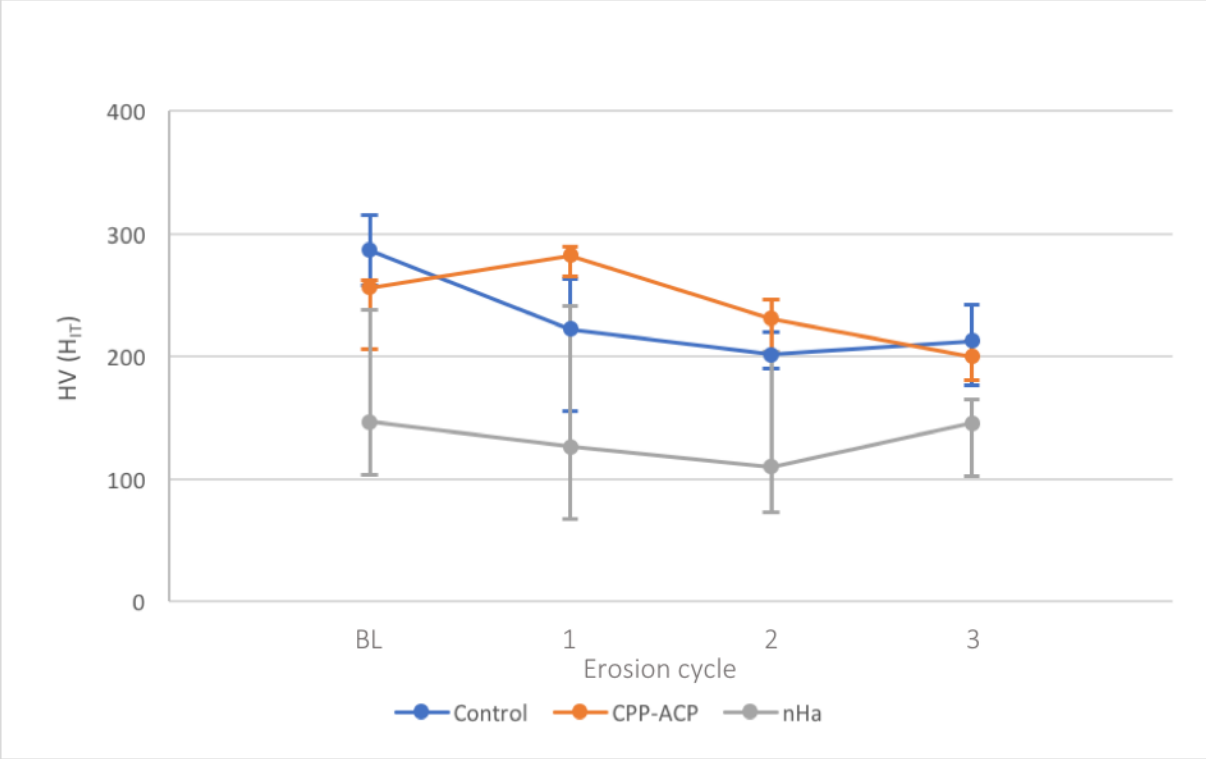


Figure 114 Median (IQR) HV values of enamel at baseline i.e. after treatment using pH9 HP and remineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Mineral composition

After exposure to three erosion cycles, atomic % of enamel calcium, phosphorus, and carbon were not significantly different between treatment groups (P>0.05) (Table 63).

	Element	Atomic %
nHA	Ca	23.5 (2.3)
	P	18.3 (1.7)
	C	30.2 (0.3)
CPP-ACP	Ca	20.0 (3.8)
	P	16.4 (1.2)
	C	26.7 (10.0)
Control	Ca	16.9 (0.2)
	P	19.3 (1.0)
	C	41.5 (3.1)

Table 63 Mean atomic % and standard deviation (SD) of eroded enamel calcium, phosphorous, and carbon after whitening using pH9 HP and remineralisation using CPP-ACP or nHA in comparison to a control group.

Surface morphology

Samples remineralised using nHA showed an irregular surface of accumulated nHA crystals, forming overlapping layers on the eroded enamel. In addition, enamel treated with CPP-ACP and control enamel samples showed an overall surface deformation, more so in the control group (Figure 115-117).

nHA

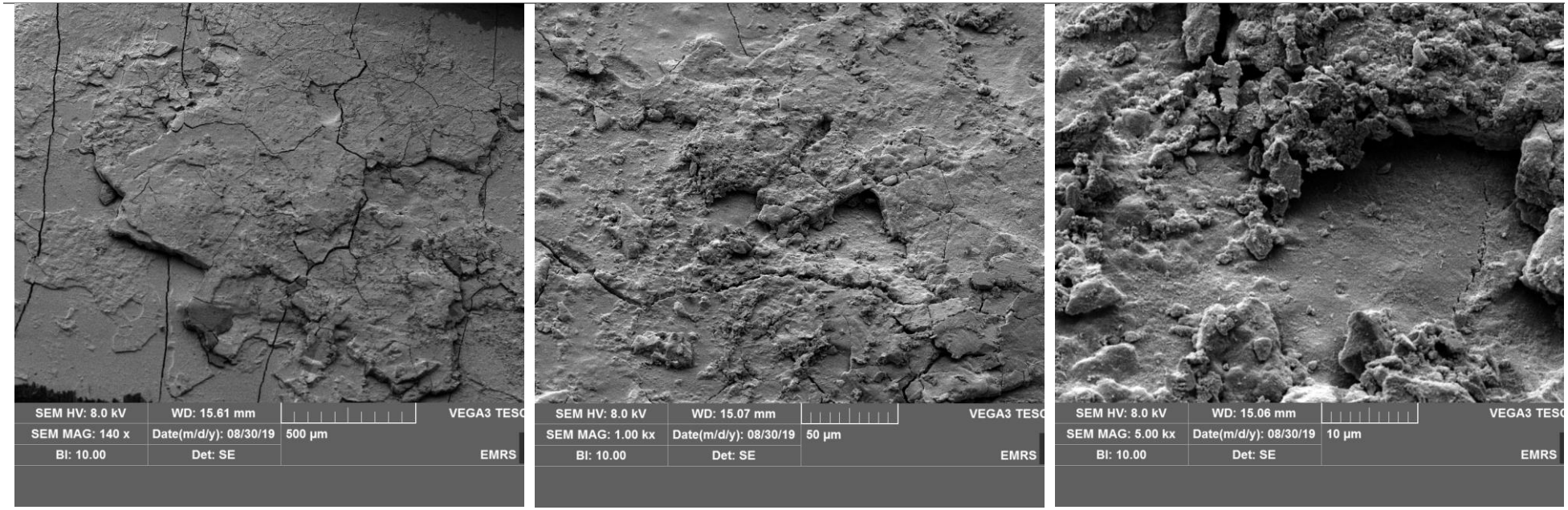


Figure 115 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP and remineralised using 15.5% nHA, then subjected to three erosion cycles. Note the accumulation of nHA crystals.

CPP-ACP

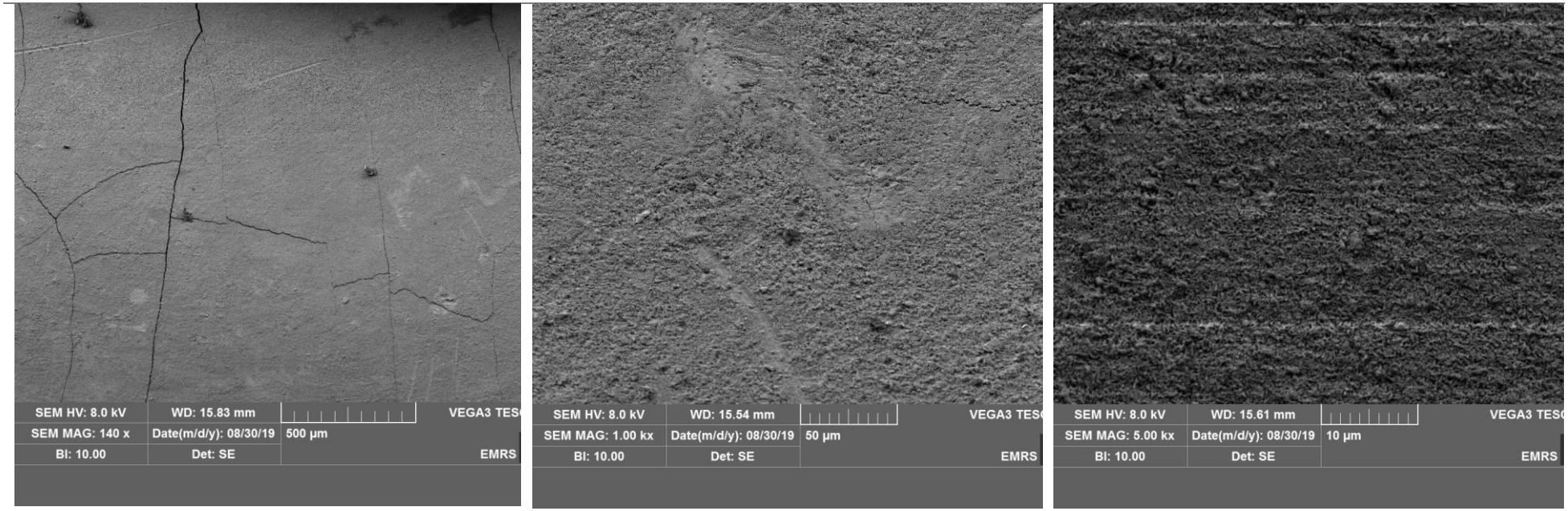


Figure 116 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP and remineralised using CPP-ACP, then subjected to three erosion cycles. Erosive changes were noted in treated enamel under all magnifications.

Control

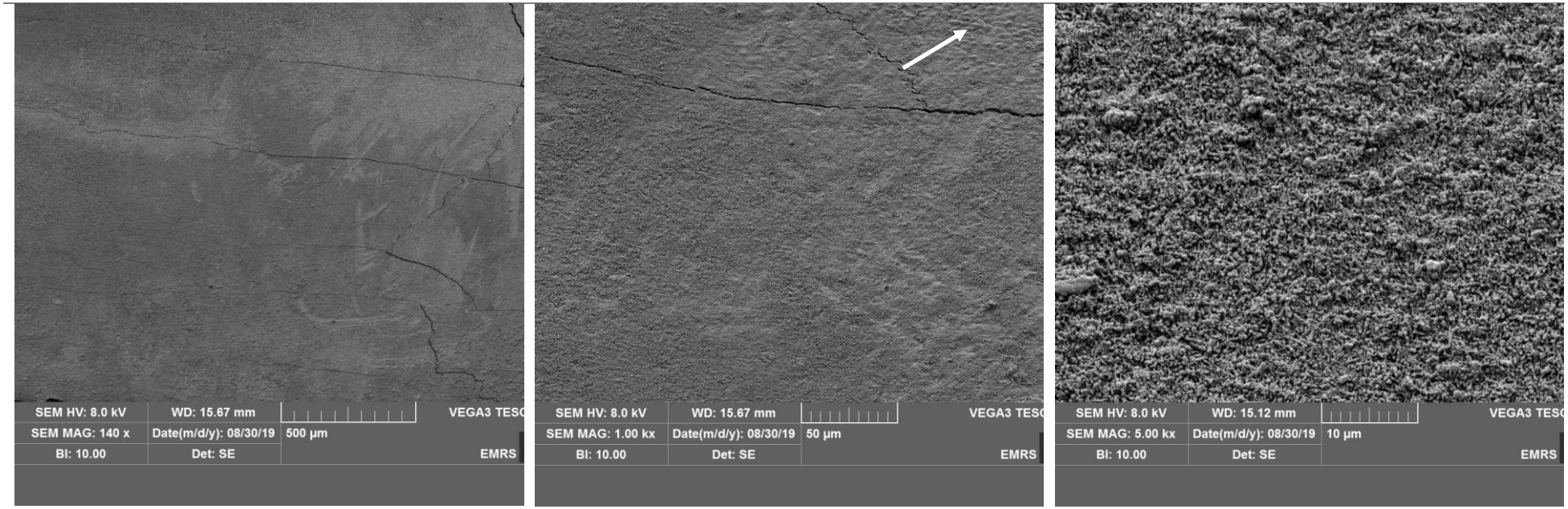


Figure 117 Three SEM images under low, medium, and high magnification of enamel samples treated with pH9 6% HP, then subjected to three erosion cycles. Erosive changes were noted in treated enamel under all magnifications. In addition, loss of interprismatic enamel can be visibly seen under medium magnification (arrow).

7.6.4 Enamel treated using PBS (Control)

Enamel surface topography

Enamel treated with CPP-ACP and control enamel samples showed a flat smooth surface at BL and after the first erosion cycle, then sharp enamel peaks were evident after the second erosion cycle, which became more rounded after the third erosion cycle (Figure 118). The nHA group, on the other hand, maintained an irregular surface with shallower valleys as erosion cycles progressed.

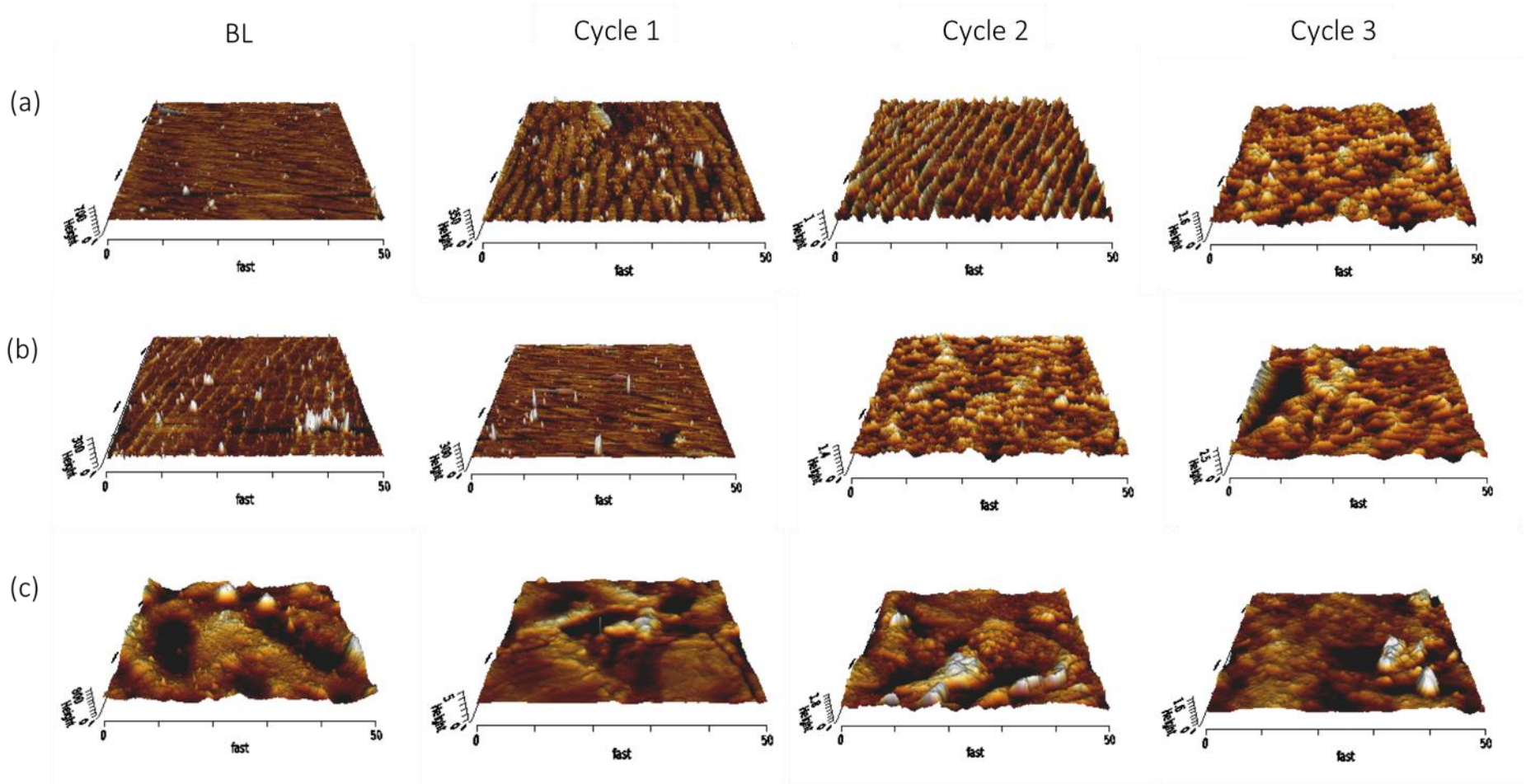


Figure 118 Representative 3D images of bovine enamel not mineralised (a), treated with CPP-ACP (b), and treated with nHA (c) at BL and after Cycle 1, Cycle 2, and Cycle 3 of erosion. Fast axis: the axis that the AFM probe scans along. Note: the z-scale changes between images due to software settings that could not be changed. The z-axis is in µm in all images captured except in group (a,b, and c) at BL and group (a) and (b) after cycle 1 which are in nm.

Enamel roughness

Control enamel and samples treated with CPP-ACP showed no significant changes in Ra after the first erosion cycle ($P>0.05$) and a significant increase in roughness after the second, and third erosion cycles ($P\leq0.05$) (Table 64) (Figure 119). Samples treated with nHA, on the other hand, showed a significant decrease in Ra after all erosion cycles in comparison to BL ($P\leq0.05$).

	Ra (nm)			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	7.0 (3.9)^{a}	6.3 (63.2)^{a}	108.0 (38.0)^{b}	116.1 (33.5)^{b}
CPP-ACP	7.8 (3.5)^{a}	5.2 (3.1)^{a}	182.0 (207.3)^{b}	351.5 (224.5)^{c}
nHA	324.6 (296.4)^{a}	128.7 (215.7)^{b}	93.7 (60.7)^{c}	120.1 (131.0)^{b}

Table 64 Median (IQR) Ra values of enamel at baseline i.e. after treatment using PBS and mineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

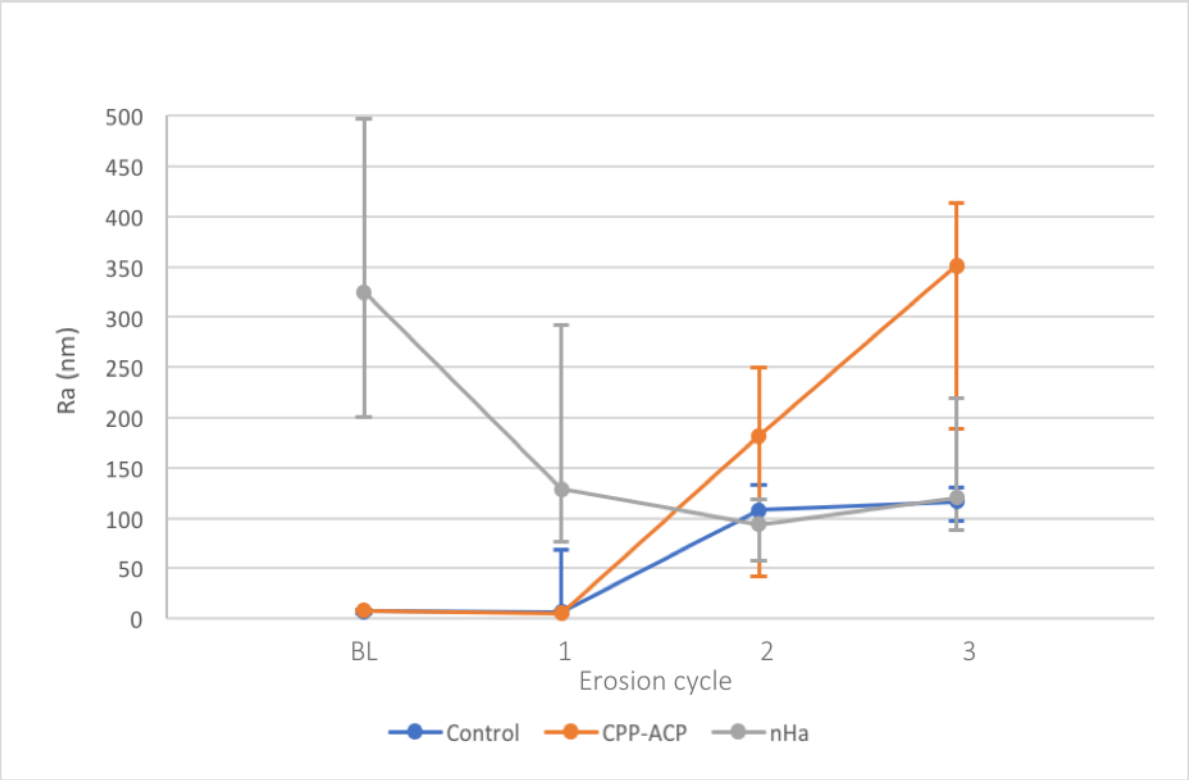


Figure 119 Median (IQR) Ra values of enamel at baseline i.e. after treatment using PBS and mineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Enamel hardness (HV)

There were no significant changes in HV after the first and second erosion cycles in control enamel samples (P>0.05). The third erosion cycle, however, resulted in a significant reduction in enamel hardness values (P≤0.05) (Table 65) (Figure 120). Enamel treated with CPP-ACP showed no significant change in HV after the first erosion cycle (P>0.05) and a significant hardness reduction was recorded after the second and third erosion cycles compared to BL values (P≤0.05). On the other hand, enamel treated using nHA showed no significant changes in hardness after all erosion cycles (P>0.05), with great variability in HV values recoded.

	HV (H_{IT})			
Treatment	BL	Cycle 1	Cycle 2	Cycle 3
Control	252.0 (55.1)[a]	185.6 (101.6)[a]	223.0 (45.4)[a]	155.3 (70.7)[b]
CPP-ACP	265.7 (55.3)[a]	227.3 (27.6)[a,b]	200.2 (32.4)[b,c]	188.2 (38.1)[c]
nHA	109.7 (85.0)[a]	161.1 (109.0)[a]	44.0 (171.6)[a]	168.7 (144.7)[a]

Table 65 Median (IQR) HV values of enamel at baseline i.e. after treatment using PBS and mineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Different superscript letters indicate significant differences between cycles.

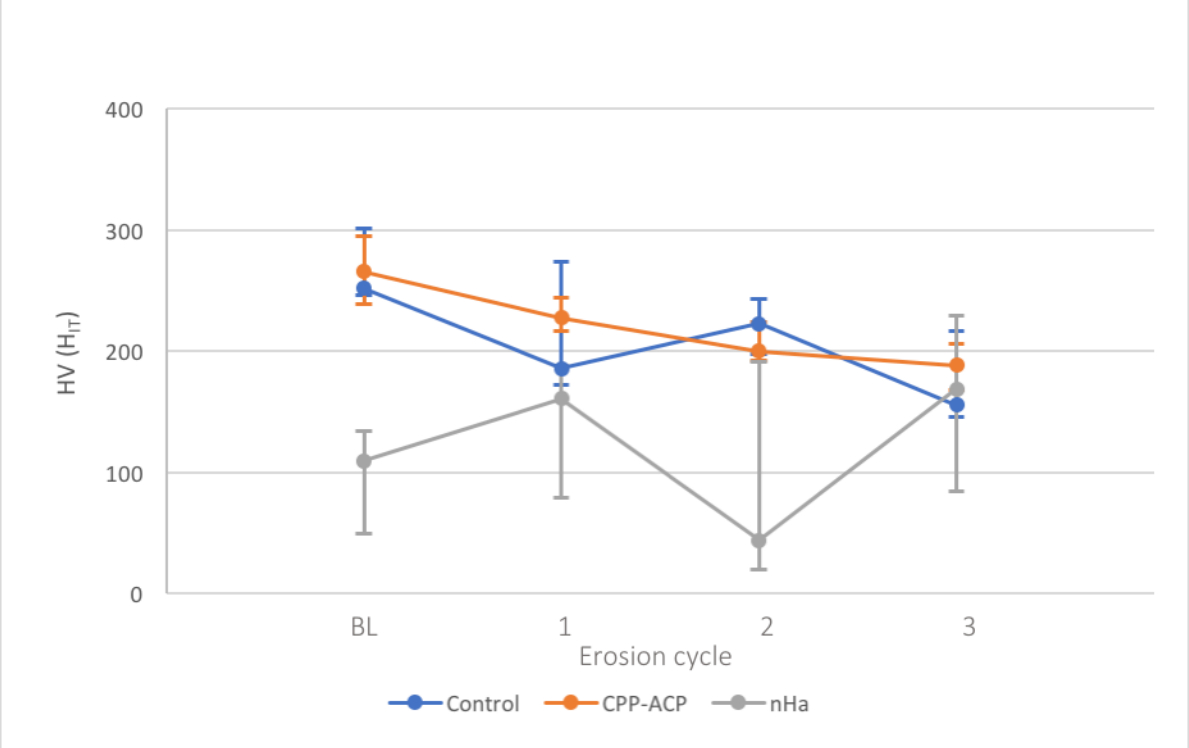


Figure 120 Median (IQR) HV values of enamel at baseline i.e. after treatment using PBS and mineralisation with either CPP-ACP or nHA or remained as a control, after the first erosion cycle, after the second erosion cycle, and after the third erosion cycle. Error bars represent the variability of data.

Mineral composition

After exposure to three erosion cycles, atomic % of enamel calcium, phosphorus, and carbon were not significantly different between treatment groups (P>0.05) (Table 66).

	Element	Atomic %
nHA	Ca	19.8 (2.0)
	P	15.1 (0.1)
	C	11.1 (2.7)
CPP-ACP	Ca	19.6 (0.5)
	P	17.1 (0.6)
	C	14.7 (2.0)
Control	Ca	21.3 (2.9)
	P	14.8 (1.1)
	C	8.8 (1.5)

Table 66 Mean atomic % and standard deviation (SD) of eroded enamel calcium, phosphorous, and carbon after treatment using PBS and mineralisation using CPP-ACP or nHA in comparison to a control group.

Surface morphology

Samples treated using nHA showed an irregular surface of accumulated nHA crystals, forming overlapping layers on the eroded enamel. In addition, enamel treated with CPP-ACP showed an irregular and deformed surface by the development of pits. Similar surface changes were noted in the eroded control enamel along with the exposure of enamel prisms (Figure 121-123).

nHA

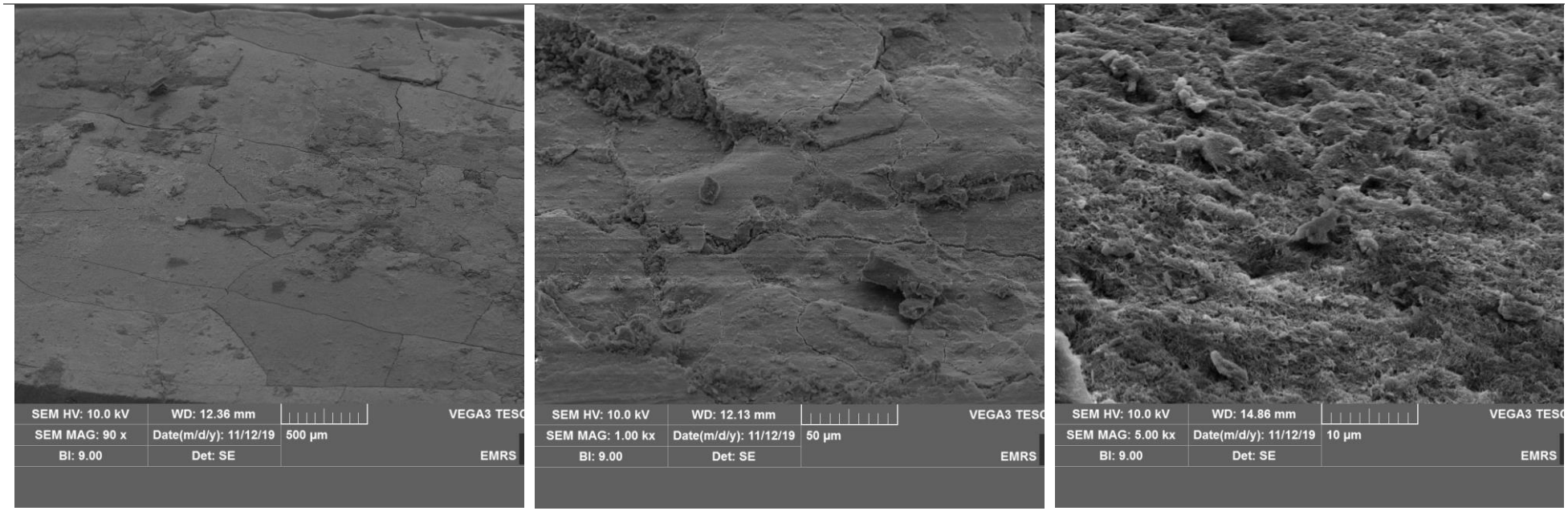


Figure 121 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS and 15.5% nHA, then subjected to three erosion cycles. Note the accumulation of nHA crystals.

CPP-ACP

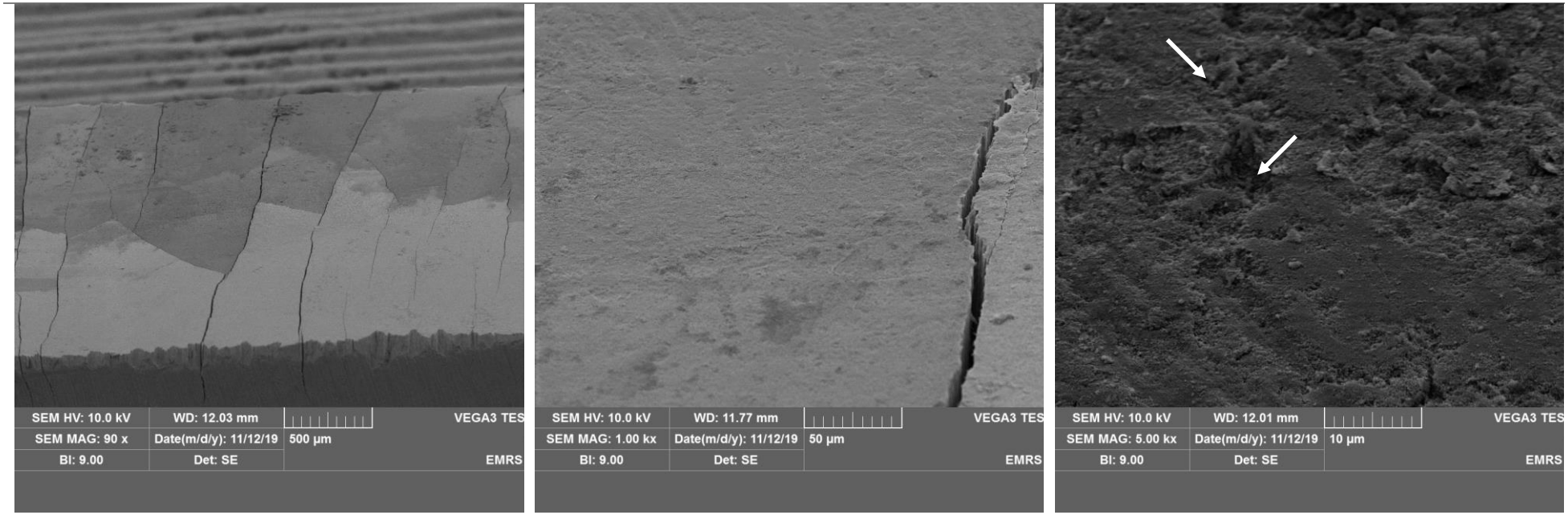


Figure 122 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS and CPP-ACP, then subjected to three erosion cycles. Erosive changes were noted by the development of pits (arrow).

Control

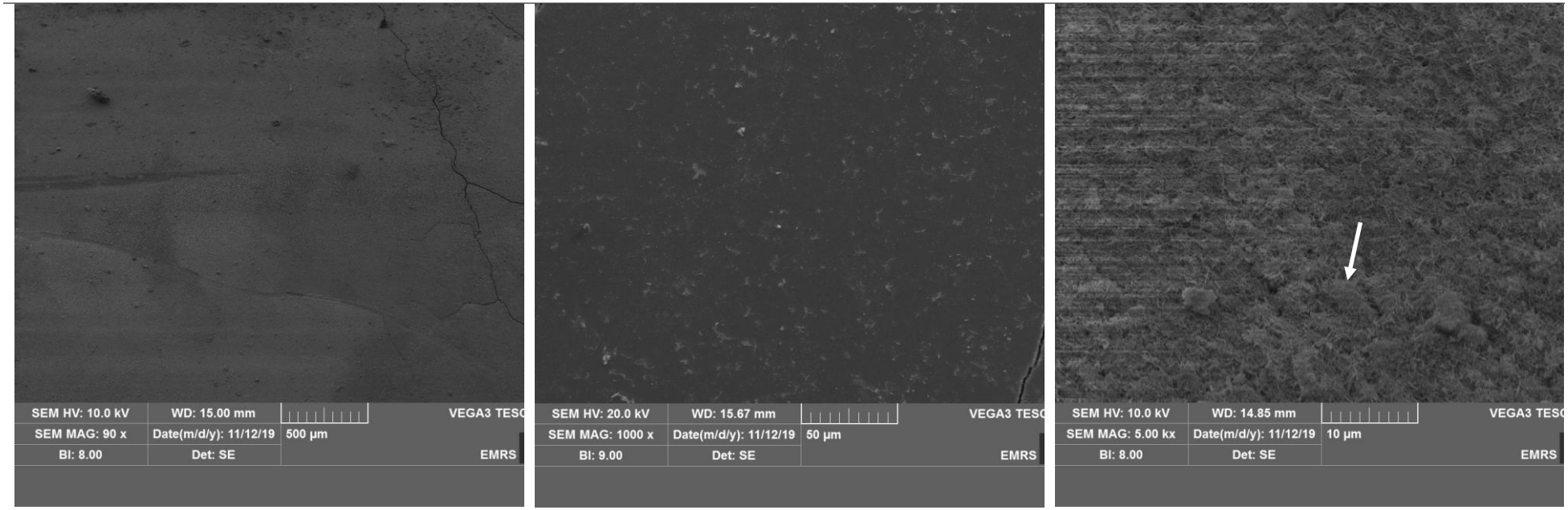


Figure 123 Three SEM images under low, medium, and high magnification of enamel samples treated with PBS, then subjected to three erosion cycles. Erosive changes by exposure of enamel prisms were noted under high magnification (arrow).

7.7 Simulated dietary staining results

7.7.1 Enamel whitened using pH5 HP

After the first staining cycle in day1, a significant increase in ΔE was recorded in enamel treated with CPP-ACP and control enamel samples (ΔE >2) (Table 67). No significant changes in ΔE were recorded after the second and third staining cycles in day1 and after all staining cycles in day2 and day3 (ΔE ≤2). Enamel treated with nHA, on the other hand, showed a significant colour change after the first staining cycle in day1, 2, and 3 (ΔE >2).

Lightness values significantly decreased after the first staining cycle in day1 in enamel treated with CPP-ACP, nHA, and control samples ($P \leq 0.05$), and values did not significantly change throughout staining cycles carried out afterwards ($P > 0.05$) (Table 68) (Figure 124-126). In addition, enamel treated with CPP-ACP and control enamel samples showed a significant increase in a* values towards the red end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) which did not significantly change after the second and third staining cycles in day1 and after all staining cycles carried out in day2 and day3 ($P > 0.05$). Enamel treated with nHA, however, showed a consistent increase in a* values towards the red end of the spectrum in day1, day2, and day3 of staining ($P \leq 0.05$). With regards to b* values, no significant changes were noted in enamel treated with CPP-ACP and control enamel samples after being subjected to all dietary staining cycles ($P > 0.05$). Enamel treated with nHA, on the other hand, showed a significant increase in b* values towards the yellow end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) and values did not significantly change afterwards ($P > 0.05$).

ΔE								
Day 1								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
4.2 (1.8)	0.8 (0.3)	1.0 (0.6)	11.4 (3.4)	1.8 (0.4)	1.4 (0.3)	2.2 (1.0)	0.9 (0.5)	1.1 (0.5)
Day 2								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
0.8 (0.4)	1.4 (0.3)	1.1 (0.5)	2.3 (1.4)	1.7 (0.9)	1.7 (0.5)	1.4 (1.0)	1.6 (0.4)	1.0 (0.4)
Day 3								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
1.0 (0.4)	0.6 (0.4)	0.7 (0.2)	3.5 (1.0)	1.4 (1.3)	1.0 (0.2)	1.8 (0.6)	1.2 (0.3)	0.9 (0.1)

Table 67 Mean (SD) ΔE values in enamel whitened using pH5 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days.

L* value										
	Day1				Day2			Day3		
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	90.1[a] (2.3)	83.8[b] (2.5)	83.5[b] (2.1)	83.7[b] (1.3)	83.3[b] (1.6)	82.7[b] (1.9)	82.4[b] (1.8)	81.9[b] (1.2)	81.7[b] (1.6)	81.2[b] (1.6)
nHA	88.4[a] (1.3)	75.9[b] (2.7)	74.3[b] (2.6)	73.2[b] (2.6)	74.1[b] (1.8)	72.7[b] (3.0)	72.2[b] (3.2)	70.8[b] (3.1)	69.5[b] (2.8)	69.1[b] (2.7)
Control	87.4[a] (1.1)	84.2[b] (1.0)	83.1[b] (0.7)	84.5[b] (0.7)	83.1[b] (1.9)	83.0[b] (1.4)	83.4[b] (1.0)	81.2[b] (1.3)	81.7[b] (1.1)	81.8[b] (0.9)
a* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	-1.3[a] (0.1)	-0.8[b] (0.1)	-0.7[b] (0.2)	-0.6[b] (0.2)	-0.5[b] (0.2)	-0.4[b] (0.3)	-0.4[b] (0.3)	-0.4[b] (0.2)	-0.3[b] (0.3)	-0.2[b] (0.3)
nHA	-1.1[a] (0.1)	1.0[b] (1.0)	1.6[b] (0.8)	2.3[b] (1.2)	1.4[c] (1.0)	1.9[c] (1.1)	2.7[c] (1.4)	2.1[c] (1.1)	2.6[c] (1.2)	2.8[c] (1.3)
Control	-1.3[a] (0.1)	-0.8[b] (0.2)	-0.7[b] (0.2)	-0.5[b] (0.2)	-0.7[b] (0.2)	-0.3[a,b] (0.3)	-0.3[b] (0.2)	-0.5[b] (0.2)	-0.4[b] (0.2)	0.2[b] (0.2)
b* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	1.6[a] (0.4)	0.8[a] (0.6)	1.2[a] (0.9)	1.6[a] (0.7)	1.5[a] (0.9)	2.2[a] (1.0)	2.4[a] (0.9)	2.0[a] (0.7)	2.3[a] (0.9)	2.4[a] (0.9)
nHA	3.0[a] (1.7)	12.8[b] (5.1)	14.0[b] (4.1)	15.3[b] (4.4)	12.4[b] (3.4)	13.7[b] (3.4)	15.7[b] (3.6)	13.5[b] (3.3)	14.6[b] (3.3)	15.1[b] (3.7)
Control	2.0[a] (1.4)	1.8[a] (1.1)	1.9[a] (0.9)	2.3[a] (0.8)	1.9[a] (1.1)	3.0[a] (1.3)	3.1[a] (0.8)	2.2[a] (1.1)	2.7[a] (0.9)	3.2[a] (0.8)

Table 68 Mean (SD) L, a*, and b* values in enamel whitened using pH5 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days. Different superscript letters indicate significant differences between staining cycles in day 1, 2, and 3. Results from each staining cycle was statistically compared to the prior staining cycle and the following staining cycle in addition to BL.*

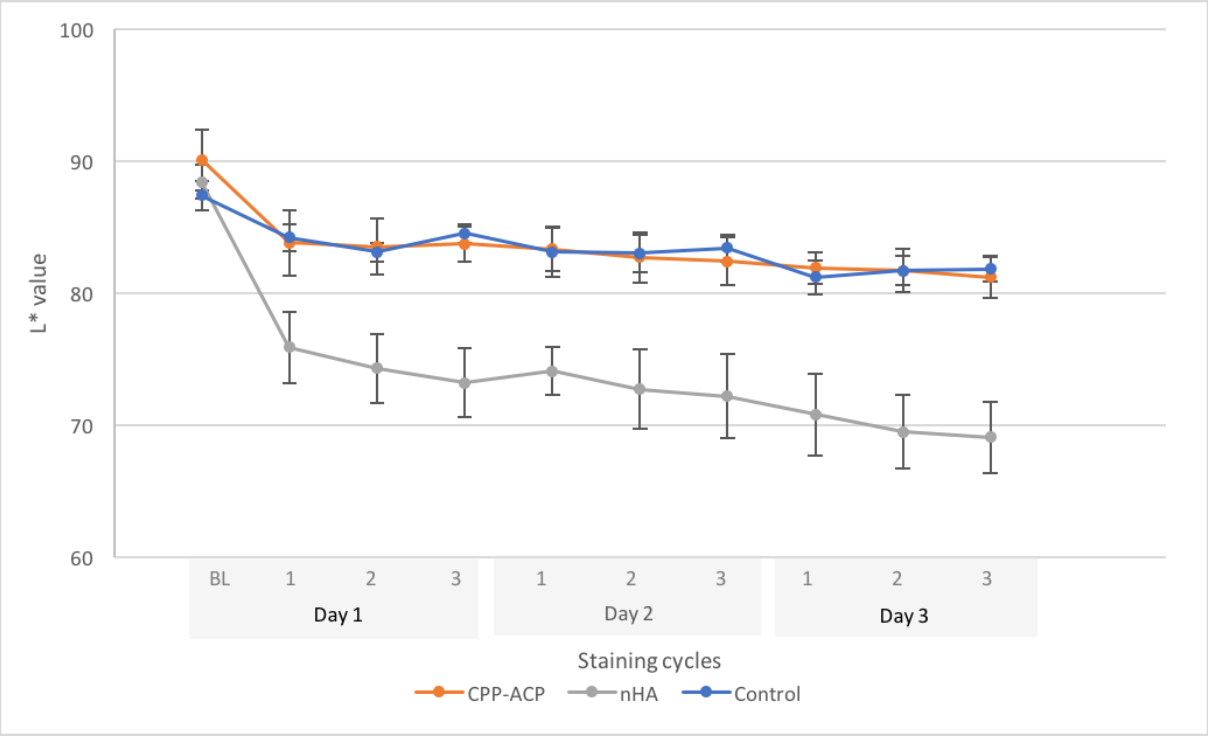


Figure 124 Mean (SD) L values in enamel whitened using pH5 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

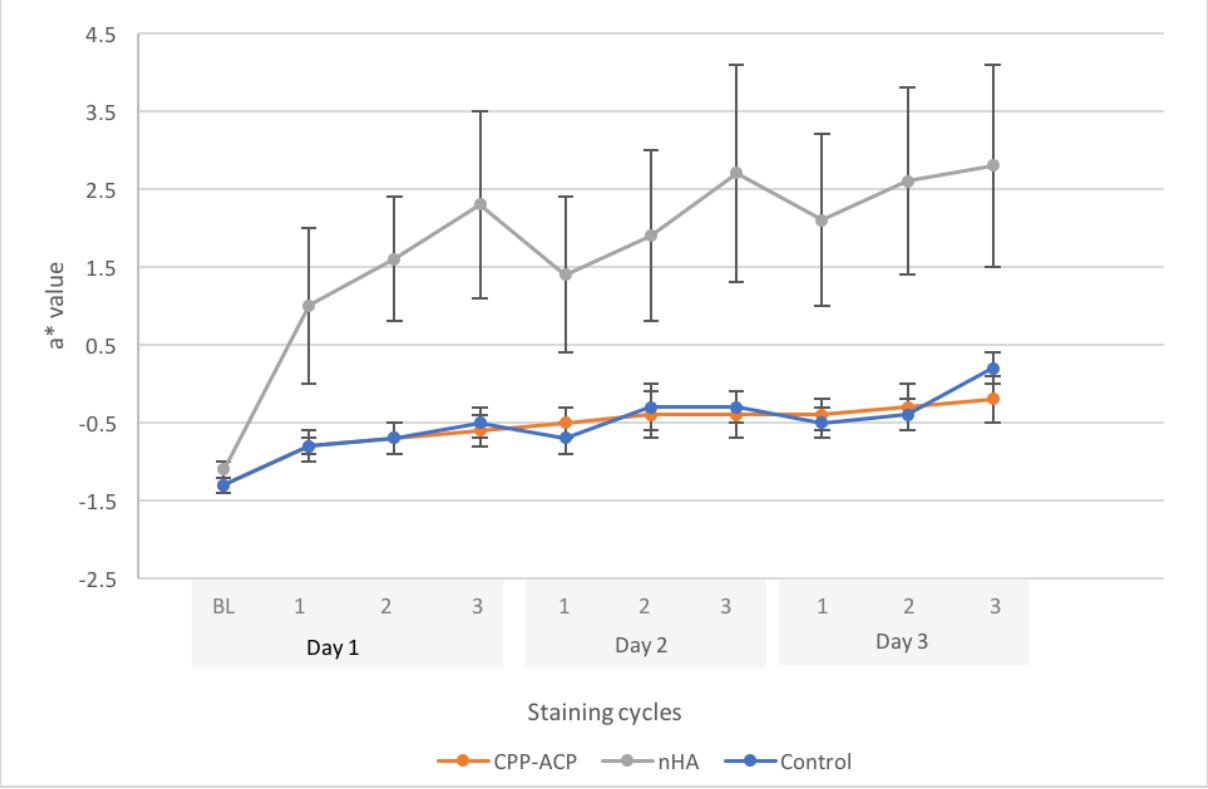


Figure 125 Mean (SD) a values in enamel whitened using pH5 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

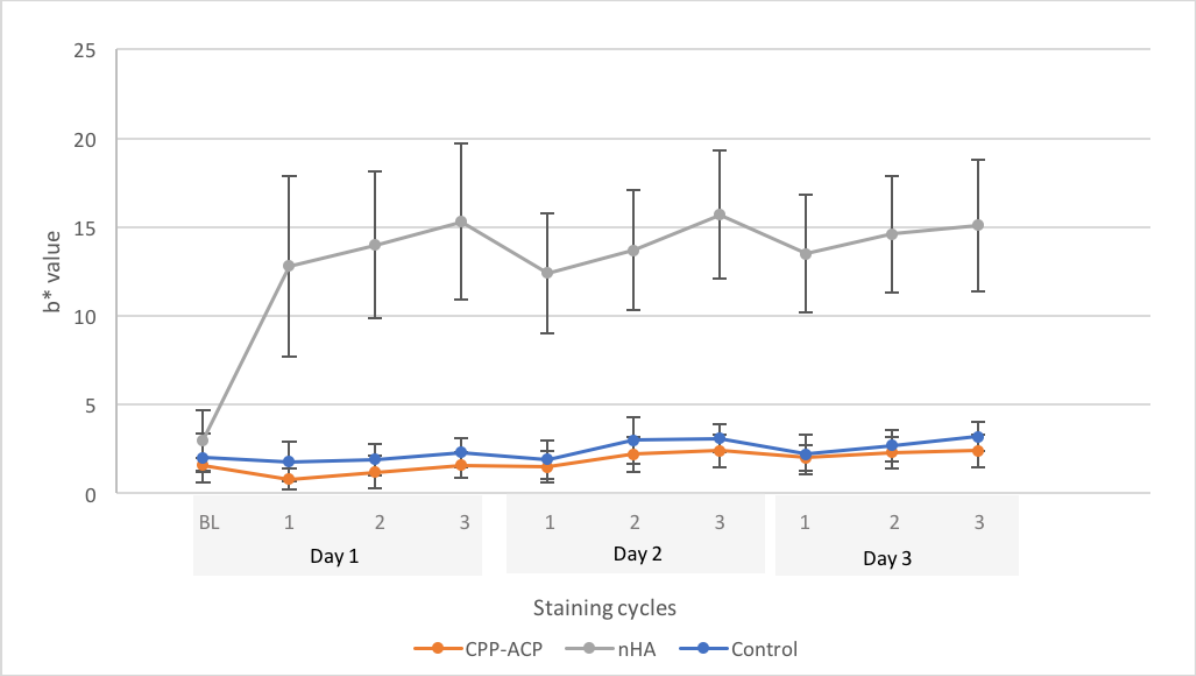


Figure 126 Mean (SD) b values in enamel whitened using pH5 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

7.7.2 Enamel whitened using pH7 HP

After the first staining cycle in day1 and day2, a significant increase in ΔE was recorded in enamel treated with CPP-ACP and control enamel samples (ΔE >2) (Table 69). No significant changes in ΔE were recorded after the second and third staining cycles in day1 and day2, and after all staining cycles in day3 (ΔE ≤2). Enamel treated with nHA, on the other hand, showed a significant colour change after the first and second staining cycles in day1 and after the first staining cycle in day2 (ΔE >2).

Lightness values significantly decreased after the first staining cycle in day1 in enamel treated with CPP-ACP ($P \leq 0.05$) (Figure 127-129). Further reductions in lightness were recorded in day2 and day3 of staining ($P \leq 0.05$) (Table 70). Enamel treated with nHA showed a significant decrease in lightness after the first and second staining cycles in day1 ($P \leq 0.05$), and values remained consistently lower throughout staining cycles carried out afterwards compared to BL ($P \leq 0.05$). Control enamel samples, on the other hand, showed no significant changes in lightness after all staining cycles in day1 ($P>0.05$). A significant decrease in lightness was recorded after the first staining cycle in day2 ($P \leq 0.05$) and again after the first staining cycle in day3 ($P \leq 0.05$) which in both cases lightness values remained the same throughout all staining cycles carried out in each day ($P>0.05$). In regard to a* values, all treatment groups showed a significant increase in a* values towards the red end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) then a* values did not significantly change after the second and third staining cycles in day1 and after all staining cycles in day2 and day3 ($P>0.05$). In regards to b* values, enamel treated with nHA showed a significant increase in b* values towards the yellow end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) which did not significantly change following all staining cycles carried out afterwards ($P>0.05$). Enamel treated with CPP-ACP showed a significant increase in b* values after the first staining cycle in day1 ($P \leq 0.05$). Values significantly declined in day2 and day3 of staining while remaining significantly higher than values recorded at BL ($P \leq 0.05$). Control enamel samples, on the other hand, showed a significant increase in b* values after the second staining cycle in day1 in comparison to BL, which remained the same following all staining cycles carried out afterwards ($P>0.05$).

ΔE								
Day1								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
4.4 (1.3)	1.1 (0.6)	0.7 (0.4)	13.5 (2.6)	4.0 (1.3)	1.5 (0.8)	3.2 (0.5)	1.7 (0.9)	2.0 (0.9)
Day2								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
2.2 (0.7)	0.3 (0.2)	0.3 (0.1)	2.9 (0.7)	1.5 (0.6)	0.8 (0.8)	2.6 (0.6)	1.0 (0.7)	0.5 (0.1)
Day3								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
1.9 (0.3)	0.7 (0.2)	0.5 (0.5)	1.7 (1.0)	1.2 (0.5)	0.7 (0.3)	1.5 (0.5)	0.8 (0.1)	0.5 (0.2)

Table 69 Mean (SD) ΔE values in enamel whitened using pH7 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days.

L* value										
	Day1				Day2			Day3		
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	88.3[a] (1.0)	85.4[b] (1.6)	84.1[b] (1.2)	83.7[b] (1.3)	80.9[c] (0.7)	80.6[c] (0.8)	80.6[c] (1.0)	78.7[b] (1.0)	79.0[b] (1.3)	78.7[b] (0.7)
nHA	87.4[a] (0.4)	77.1[b] (3.0)	72.2[c] (2.3)	70.8[c] (3.5)	68.8[c] (4.0)	67.5[c] (3.6)	66.9[c] (4.2)	66.6[c] (4.3)	65.7[c] (4.4)	65.2[c] (4.3)
Control	85.4[a,c] (0.5)	88.4[b] (1.0)	86.4[a] (1.1)	84.1[c] (1.7)	80.7[d] (1.6)	80.5[d] (1.5)	80.6[d] (1.3)	79.1[b] (0.8)	78.8[b] (1.0)	78.8[b] (0.7)
a* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	-1.4[a] (0.1)	0.1[b] (0.3)	0.3[b,c] (0.2)	0.5[c] (0.2)	0.4[c] (0.1)	0.5[c] (0.2)	0.7[c] (0.1)	0.4[c] (0.1)	0.7[c] (0.1)	0.7[c] (0.1)
nHA	-1.2[a] (0.1)	3.0[b] (1.1)	4.1[b] (1.0)	4.8[b] (1.5)	3.8[b] (1.4)	4.4[b] (1.3)	4.7[b] (1.5)	3.9[b] (1.7)	4.3[b] (1.6)	4.7[b] (1.7)
Control	-1.3[a] (0.1)	-0.2[b] (0.2)	-0.04[b] (0.4)	0.3[b] (0.4)	0.5[b] (0.9)	0.4[b] (0.4)	0.4[b] (0.5)	0.3[b] (0.3)	0.5[b] (0.4)	0.6[b] (0.4)
b* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	2.3[a] (0.8)	6.2[b] (0.5)	6.6[b] (0.5)	7.0[b] (0.4)	5.7[c] (1.0)	6.0[c] (1.1)	6.2[c] (1.1)	4.5[b] (1.1)	5.0[b] (1.5)	5.1[b] (1.4)
nHA	3.5[a] (0.8)	18.9[b] (3.6)	20.4[b] (3.4)	21.4[b] (4.0)	17.0[b] (4.8)	18.4[b] (3.9)	18.6[b] (4.0)	16.3[b] (4.7)	17.5[b] (4.4)	18.0[b] (4.3)
Control	1.9[a] (0.3)	3.8[a,b] (1.1)	5.2[b,c] (1.4)	6.4[c] (1.3)	5.6[c] (0.3)	6.0[c] (0.7)	6.3[c] (0.7)	5.1[c] (0.5)	5.3[c] (0.8)	5.4[c] (0.8)

Table 70 Mean (SD) L, a*, and b* values in enamel whitened using pH7 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days. Different superscript letters indicate significant differences between staining cycles in days 1, 2, and 3. Results from each staining cycle was statistically compared to the prior staining cycle and the following staining cycle in addition to BL.*

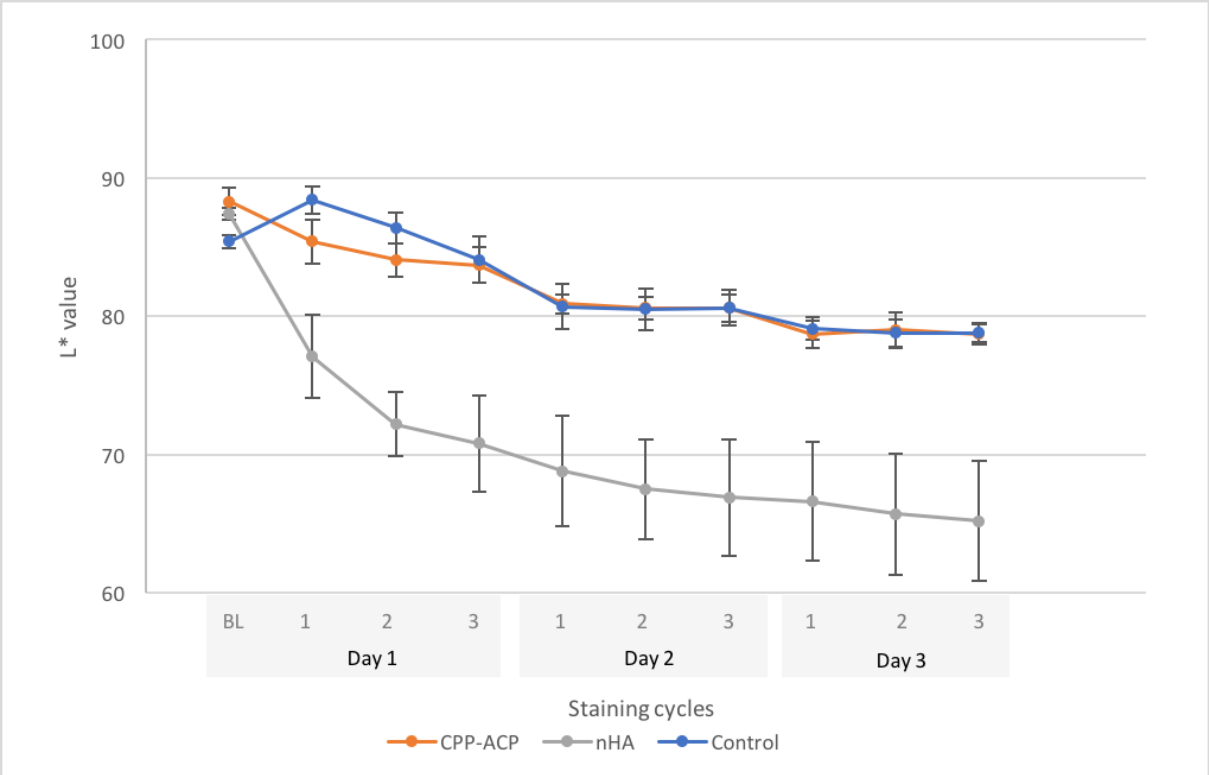


Figure 127 Mean (SD) L values in enamel whitened using pH7 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

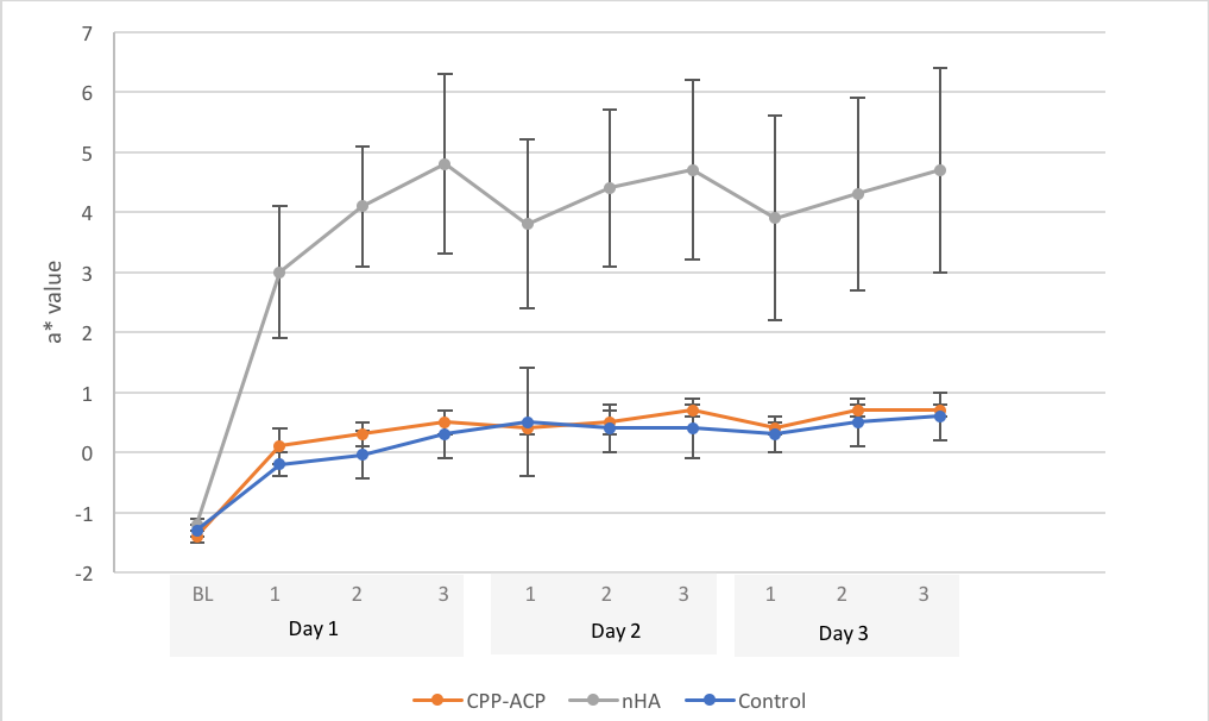


Figure 128 Mean (SD) a values in enamel whitened using pH7 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

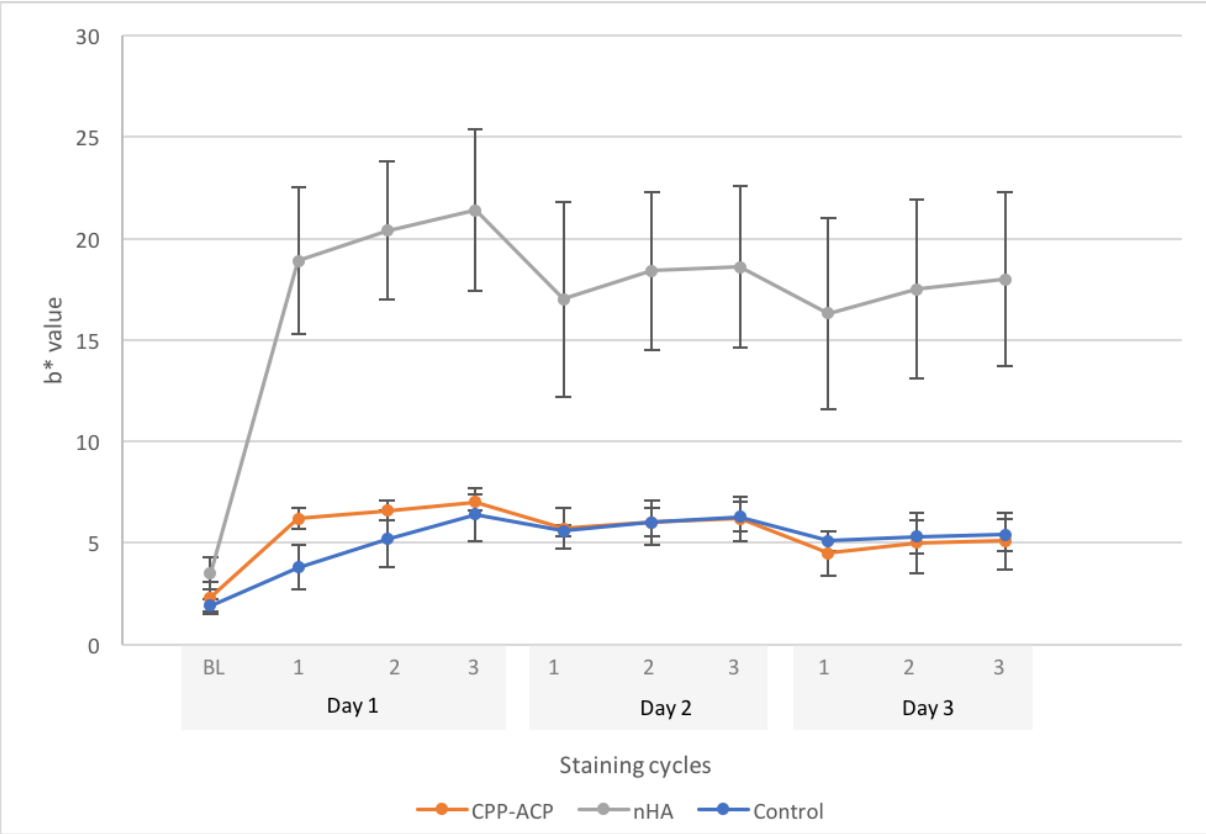


Figure 129 Mean (SD) b values in enamel whitened using pH7 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

7.7.3 Enamel whitened using pH9 HP

After the first and second staining cycles in day1, a significant increase in ΔE was recorded in enamel treated with nHA (ΔE >2) (Table 71). No significant changes in ΔE were recorded after the third staining cycle in day1 and after all staining cycles in day2 and day3 (ΔE ≤2). Enamel treated with CPP-ACP showed a significant colour change after the first staining cycle in day1, after the second and third staining cycles in day2, and after the first and second staining cycles in day3 (ΔE >2). In addition, control enamel samples showed a significant increase in ΔE after the first staining cycle in day1 and after the first and second staining cycles in day2 and day3 (ΔE >2).

Lightness values significantly decreased after the second staining cycle in day1 then again after the second staining cycle in day2 in enamel treated with CPP-ACP ($P \leq 0.05$) (Table 72) (Figure 130-132) and lightness values did not significantly change following staining cycles carried out afterwards ($P>0.05$). In addition, lightness values significantly decreased after the first staining cycle in day1 in enamel treated with nHA and after the third staining cycle in day1 in control enamel samples ($P \leq 0.05$), and in both cases L* values remained consistently lower throughout staining cycles carried out afterwards compared to BL ($P \leq 0.05$). In regards to a* values, all treatment groups showed a significant increase in a* values towards the red end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) then values did not significantly change after the second and third staining cycles in day1 and after all staining cycles in day2 and day3 ($P>0.05$). Enamel treated with nHA showed a significant increase in b* values towards the yellow end of the spectrum after the first staining cycle in day1 ($P \leq 0.05$) which did not significantly change following all staining cycles carried out afterwards ($P>0.05$). Enamel treated with CPP-ACP, however, showed fluctuating b* values after staining indicating significant stain uptake ($P>0.05$) at times or no significant differences compared to values recorded at BL ($P>0.05$). Control enamel samples showed a significant increase in b* values after the second staining cycle in day1 ($P \leq 0.05$), which did not significantly change following all staining cycles carried out afterwards ($P>0.05$).

ΔE								
Day1								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
3.4 (0.8)	1.8 (0.5)	0.9 (0.5)	15.5 (3.0)	2.5 (0.8)	1.9 (0.6)	3.8 (1.4)	1.6 (0.5)	1.0 (0.6)
Day2								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
1.6 (1.1)	2.1 (1.5)	2.3 (1.8)	1.4 (0.6)	1.5 (0.4)	1.1 (0.4)	2.1 (0.7)	3.1 (1.2)	1.4 (0.6)
Day3								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
3.8 (2.2	3.1 (1.7)	0.6 (0.3)	1.4 (0.6)	1.7 (1.1)	1.5 (0.7)	2.6 (1.2)	2.5 (1.2)	1.5 (0.7)

Table 71 Mean (SD) ΔE values in enamel whitened using pH9 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days.

L* value										
	Day1				Day2			Day3		
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	88.3^{a} (0.6)	86.3^{a,b} (1.0)	83.7^{c} (0.9)	84.3^{a,c} (1.9)	84.5^{a,b} (3.0)	81.8^{b} (2.8)	79.9^{b} (2.6)	83.7^{b} (2.0)	80.0^{b} (2.5)	79.7^{b} (2.8)
nHA	88.1^{a} (0.9)	75.5^{b} (3.6)	72.6^{b} (3.7)	70.1^{b} (3.2)	69.8^{b} (3.8)	67.9^{b} (3.9)	67.0^{b} (3.6)	68.0^{b} (5.0)	66.2^{b} (3.4)	64.4^{b} (4.0)
Control	88.6^{a} (1.0)	87.6^{a} (2.4)	85.5^{a,b} (1.9)	84.3^{b} (1.4)	86.9^{b} (2.2)	83.9^{b} (3.3)	84.5^{b} (2.6)	87.1^{b} (2.4)	83.9^{b} (3.7)	82.1^{b} (3.7)
a* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	-1.4^{a} (0.1)	-0.1^{b} (0.1)	0.05^{b} (0.2)	0.2^{b} (0.1)	0.6^{b} (0.3)	0.9^{b} (0.4)	1.0^{b} (0.3)	1.04^{b} (0.3)	1.5^{b} (0.5)	1.6^{b} (0.5)
nHA	-1.4^{a} (0.1)	2.8^{b} (1.2)	3.9^{b} (1.2)	4.4^{b} (1.4)	5.2^{b} (1.3)	5.4^{b} (1.4)	5.6^{b} (1.6)	6.3^{b} (1.8)	6.5^{b} (1.5)	6.6^{b} (1.4)
Control	-1.5^{a} (0.1)	-0.3^{b} (0.3)	-0.1^{b} (0.4)	-0.004^{b} (0.5)	-0.02^{b} (0.4)	0.4^{b} (0.7)	0.5^{b} (0.8)	0.3^{b} (0.6)	0.7^{b} (0.9)	0.9^{b} (0.9)
b* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	3.1^{a} (0.6)	6.0^{b} (1.0)	6.6^{b} (1.2)	6.9^{a} (1.0)	7.2^{a,b} (1.9)	8.5^{b} (1.3)	8.1^{a,b} (4.4)	7.3^{a,b} (1.4)	9.3^{b} (1.3)	9.6^{b} (1.4)
nHA	3.2^{a} (1.1)	20.5^{b} (2.9)	22.1^{b} (3.4)	22.6^{b} (3.5)	23.4^{b} (2.8)	23.5^{b} (3.1)	24.2^{b} (4.3)	24.3^{b} (4.1)	23.9^{b} (2.9)	23.9^{b} (3.0)
Control	2.4^{a} (0.3)	4.7^{a,b} (1.8)	5.6^{b} (1.7)	6.2^{b} (1.3)	5.5^{b} (1.9)	8.1^{b} (1.6)	7.6^{b} (1.9)	5.3^{a,b} (1.7)	6.8^{b} (2.4)	7.9^{b} (2.5)

Table 72 Mean (SD) L, a*, and b* values in enamel whitened using pH9 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days. Different superscript letters indicate significant differences between staining cycles in days 1, 2, and 3. Results from each staining cycle was statistically compared to the prior staining cycle and the following staining cycle in addition to BL.*

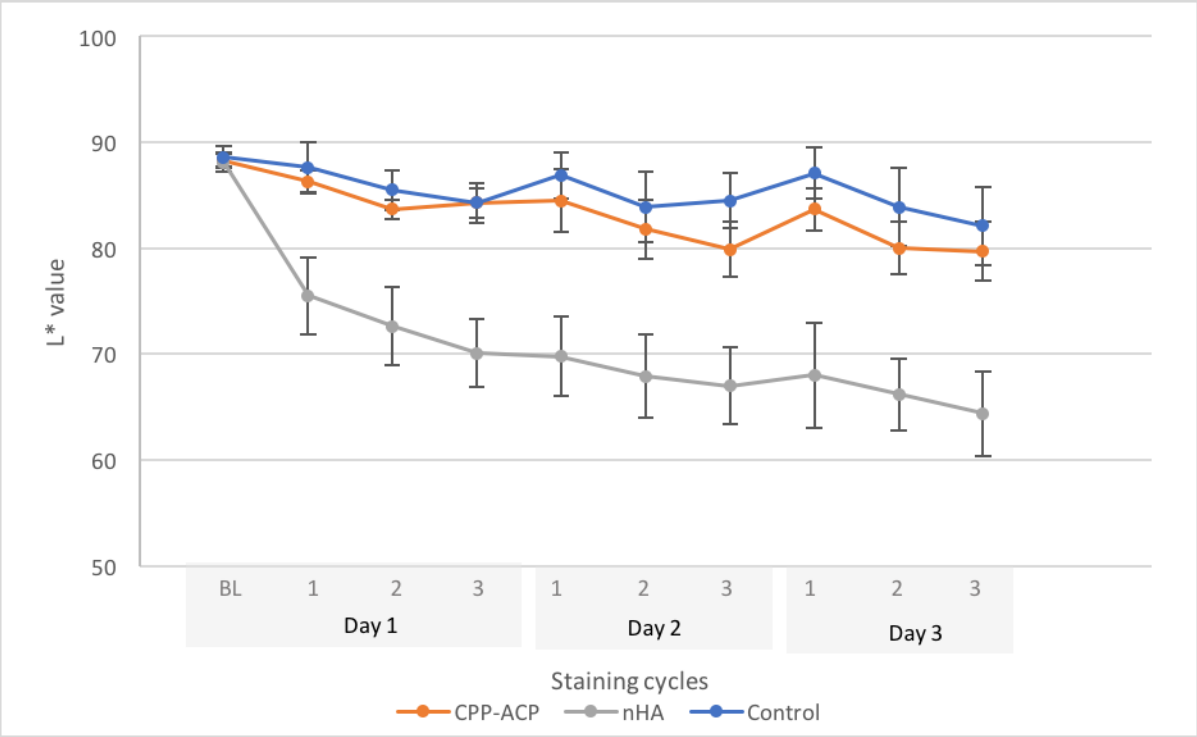


Figure 130 Mean (SD) L values in enamel whitened using pH9 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

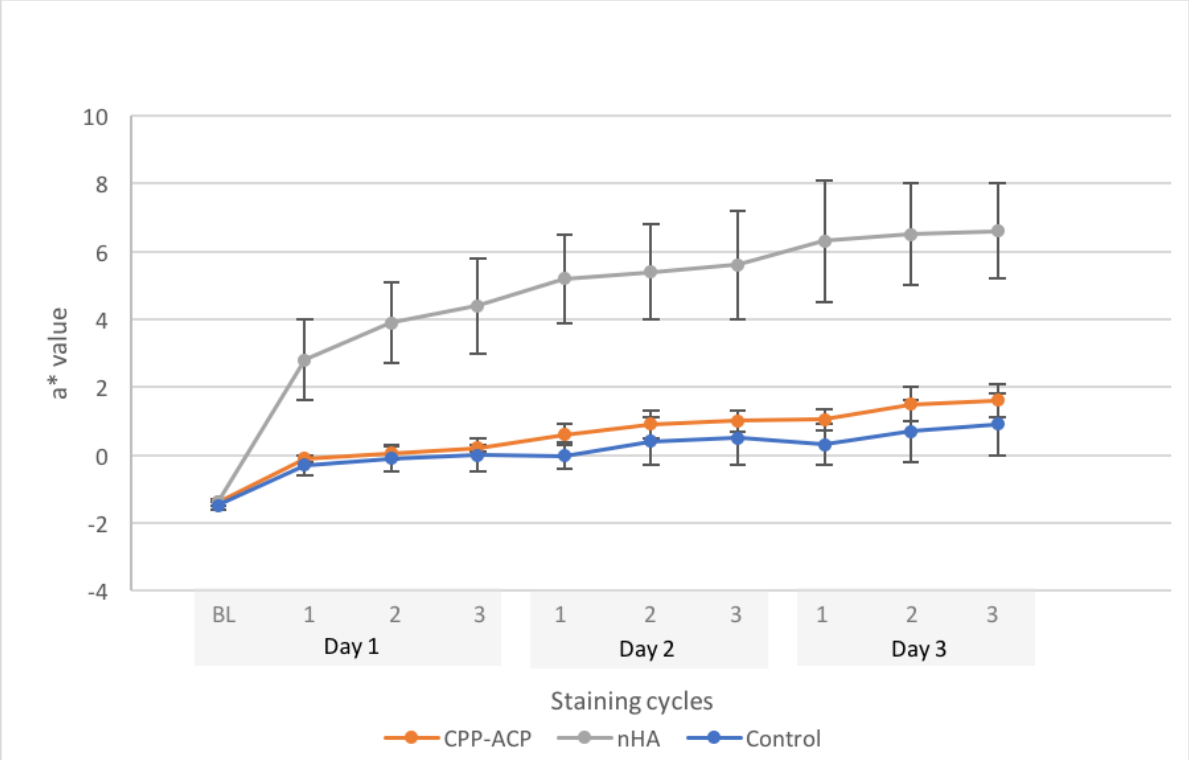


Figure 131 Mean (SD) a values in enamel whitened using pH9 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

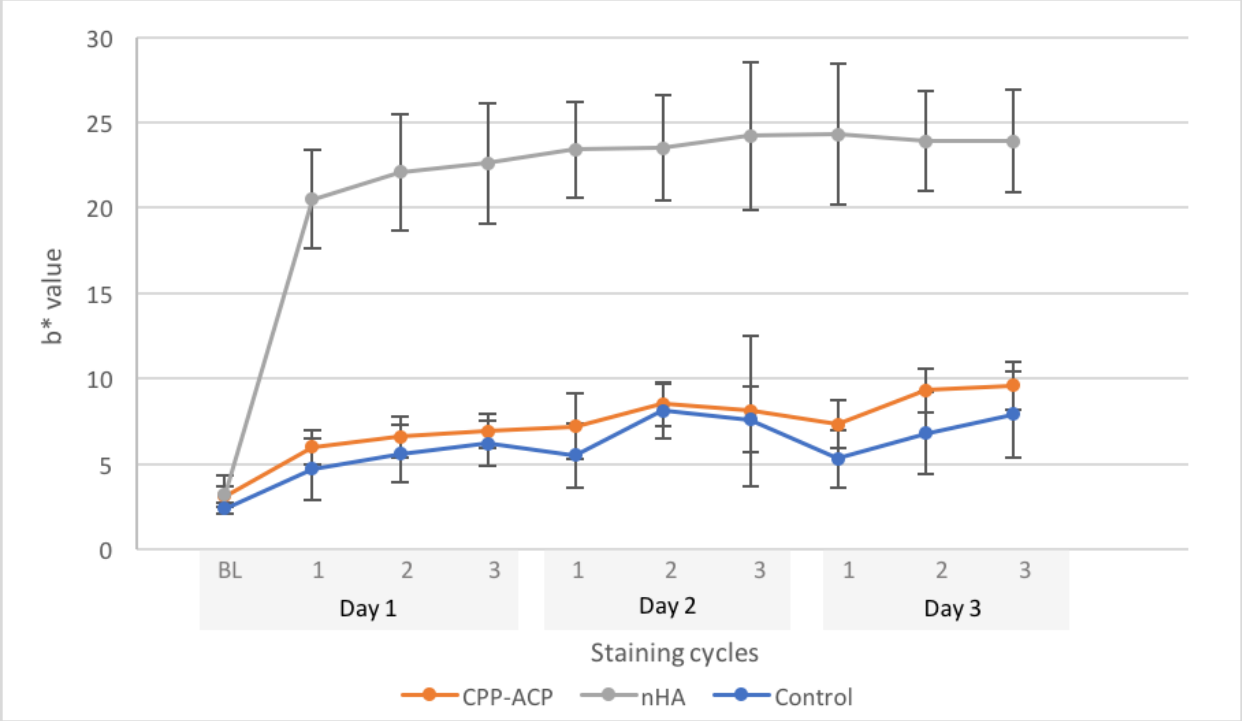


Figure 132 Mean (SD) b values in enamel whitened using pH9 HP (control) and samples whitened and remineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

7.7.4 Enamel treated using PBS (Control)

Significant changes in recorded ΔE (defined as ΔE >2) mostly occurred in enamel treated with nHA followed by CPP-ACP treated enamel, then unmineralised control enamel (Table 73). In samples treated with CPP-ACP enamel lightness did not significantly change after staining cycles carried out in day1 ($P > 0.05$) (Table 74) (Figure 133-135). In day2, however, a significant reduction in L* occurred after the third staining cycle ($P \leq 0.05$). Values did not significantly differ after the first and second staining cycles in day3 compared to BL ($P > 0.05$), then a significant reduction in enamel lightness was recorded after the third staining cycle ($P \leq 0.05$). Similarly, control enamel samples showed no significant changes in lightness after staining cycles carried out in day1 ($P > 0.05$) then significantly lower lightness values were recorded after the second staining cycle in day2 ($P \leq 0.05$) which did not significantly change throughout staining cycles carried out afterwards ($P > 0.05$). Enamel treated with nHA, on the other hand, exhibited a significant reduction in L* after the first staining cycle in day1 ($P \leq 0.05$) which was maintained throughout day1, day2, and day3 of staining. With regards to a* values, a significant increase occurred following the second staining cycle in day1 in enamel treated with CPP-ACP and control enamel samples. Same level of increase in a* was maintained afterwards in both groups ($P > 0.05$). In addition, enamel treated with nHA, showed a significant increase in a* and b* values after the first staining cycle in day1 ($P \leq 0.05$) and values did not significantly change throughout day1, day2, and day3 of staining ($P > 0.05$). Enamel treated with CPP-ACP, showed no significant changes in b* values after all staining cycles carried out in day1, day2, and day3 ($P > 0.05$) except following the third staining cycle in day2 in comparison to BL ($P \leq 0.05$). Control enamel samples, however, showed a significant increase in b* values after the second staining cycle in day1 ($P \leq 0.05$) and values were maintained throughout day1, day2, and day3 of staining ($P > 0.05$).

ΔE								
Day1								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
3.3 (0.8)	2.8 (1.1)	2.0 (1.4)	11.9 (5.9)	5.2 (4.2)	2.3 (1.0)	2.1 (0.8)	2.5 (0.7)	1.4 (0.6)
Day2								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
1.5 (0.8)	4.8 (1.4)	4.3 (3.3)	2.3 (0.4)	3.8 (1.3)	1.0 (1.1)	2.3 (1.6)	4.2 (1.1)	1.9 (0.9)
Day3								
CPP-ACP			nHA			Control		
1	2	3	1	2	3	1	2	3
5.0 (2.9)	3.6 (1.6)	3.3 (0.6)	1.0 (0.3)	3.3 (1.3)	3.9 (2.3)	5.8 (2.1)	2.7 (1.6)	4.0 (3.0)

Table 73 Mean (SD) ΔE values in enamel treated with PBS (control) and samples treated and mineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days.

L* value										
	Day1				Day2			Day3		
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	78.6[a,c] (1.2)	82.8[a] (0.2)	80.1[a] (1.0)	78.2[c] (0.5)	78.6[c] (1.3)	77.8[c,b] (5.1)	74.7[b] (1.7)	80.6[a] (3.3)	76.0[c] (1.7)	71.9[b] (1.3)
nHA	78.6[a] (0.9)	70.1[b] (5.2)	64.7[b] (3.0)	63.0[b] (4.0)	64.5[b] (4.3)	60.1[b] (3.7)	59.3[b] (4.4)	60.2[b] (5.2)	59.9[b] (4.6)	57.7[b] (5.2)
Control	79.0[a,b] (0.7)	81.3[a] (0.7)	78.3[b] (1.0)	77.3[b] (2.3)	79.7[b] (2.4)	74.4[c] (1.7)	73.0[c] (1.7)	78.2[c] (2.0)	75.8[c] (2.7)	71.0[c] (2.7)
a* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	0.6[a] (1.1)	1.2[a,b] (0.5)	2.1[b] (0.7)	2.4[b] (0.6)	2.1[a,b] (0.3)	2.5[b] (1.4)	3.4[b] (0.7)	2.3[b] (1.1)	3.5[b] (0.9)	8.3[b] (8.2)
nHA	-0.3[a] (0.9)	5.2[b] (2.0)	7.3[b] (1.1)	7.9[b] (1.7)	8.2[b] (1.5)	9.1[b] (1.4)	8.9[b] (1.6)	9.2[b] (1.6)	9.4[b] (1.6)	9.5[b] (1.5)
Control	0.7[a] (0.6)	1.3[a,b] (0.3)	2.0[b] (0.5)	2.2[b] (0.3)	2.2[b] (0.6)	3.3[b] (0.8)	3.8[b] (0.7)	3.0[b] (0.7)	3.4[b] (1.0)	4.3[b] (0.7)
b* value										
	BL	1	2	3	1	2	3	1	2	3
CPP-ACP	9.6[a] (1.0)	9.9[a] (2.0)	12.0[a] (2.6)	12.7[a] (2.3)	13.2[a] (1.8)	15.5[a,b] (5.8)	15.5[b] (2.9)	11.3[a,b] (3.4)	12.6[a] (3.8)	14.1[a] (3.9)
nHA	8.4[a] (1.0)	24.8[b] (5.1)	27.6[b] (1.8)	27.1[b] (2.5)	26.6[b] (1.4)	27.8[b] (1.6)	27.1[b] (2.3)	26.7[b] (2.9)	27.2[b] (2.3)	25.4[b] (2.7)
Control	9.7[a] (0.8)	11.0[a,b] (0.8)	12.2[b] (1.2)	12.7[b] (0.9)	12.4[b] (1.8)	14.9[b] (1.6)	16.4[b] (1.9)	13.9[b] (2.4)	13.4[b] (1.9)	16.0[b] (2.5)

Table 74 Mean (SD) L, a*, and b* values in enamel treated with PBS (control) and samples treated and mineralised with CPP-ACP or nHA. Samples were subjected to three 5-minute staining cycles, repeated for three consecutive days. Different superscript letters indicate significant differences between staining cycles in days 1, 2, and 3. Results from each staining cycle was statistically compared to the prior staining cycle and the following staining cycle in addition to BL.*

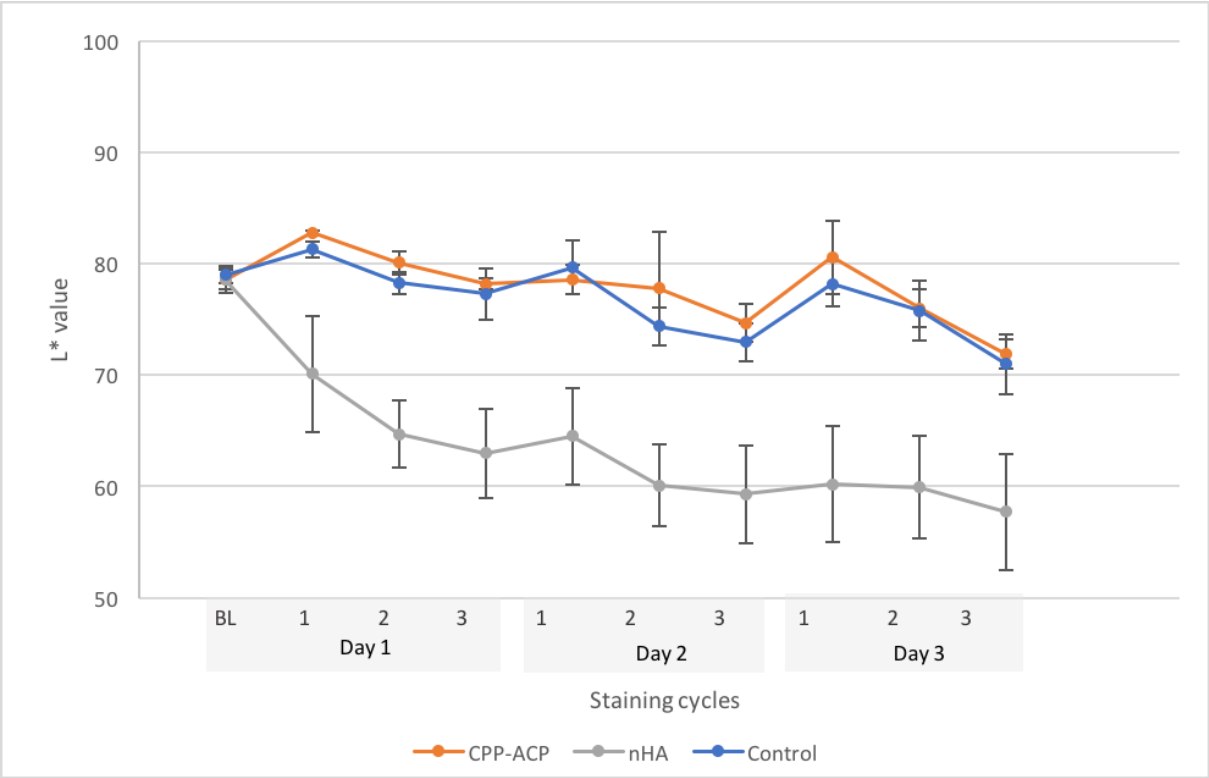


Figure 133 Mean (SD) L values in enamel treated using PBS (control) and samples treated using PBS and mineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

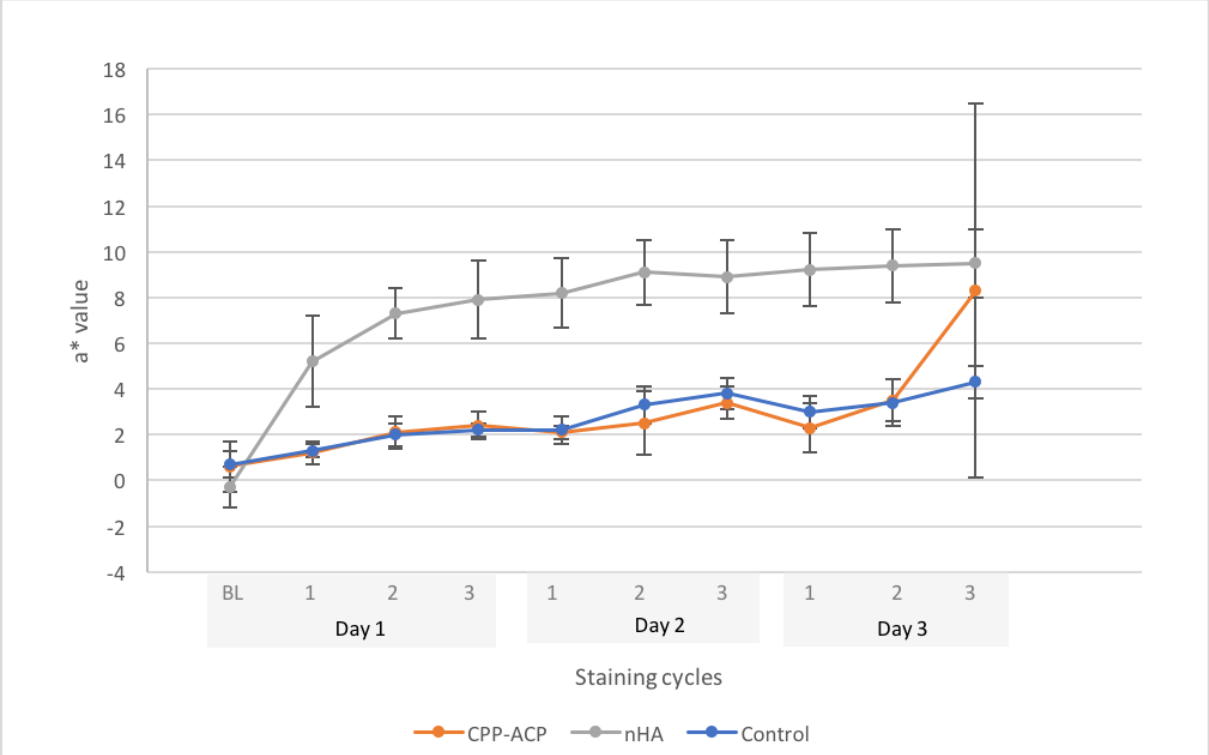


Figure 134 Mean (SD) a values in enamel treated using PBS (control) and samples treated using PBS and mineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

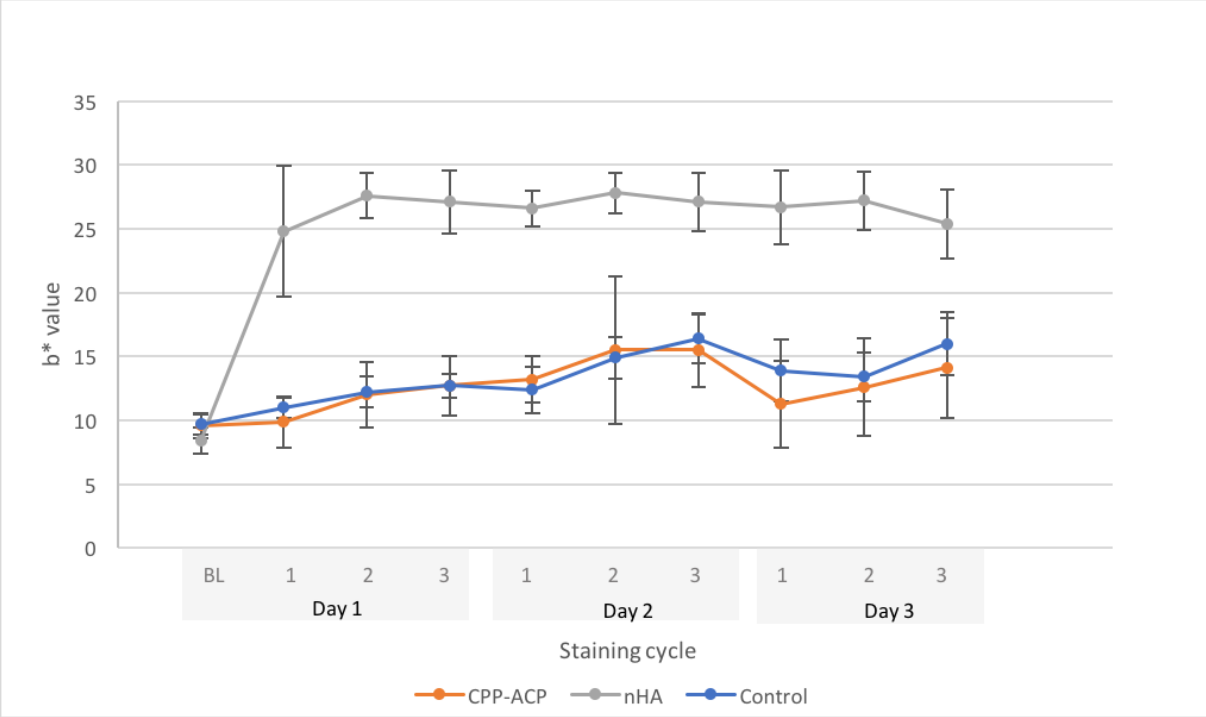


Figure 135 Mean (SD) b values in enamel treated using PBS (control) and samples treated using PBS and mineralised with CPP-ACP or nHA. Error bars represent the variability of data.*

7.8 Discussion

To date, research mostly concentrates on the remineralising abilities of various products on eroded enamel rather than studying their ability to prevent erosive damage in whitened enamel (Hegde and Moany, 2012; Bajaj *et al.*, 2016). In addition, many studies investigating the susceptibility of whitened/remineralised enamel to staining apply the remineralising agents following whitening treatments (Singh *et al.*, 2010; Públio *et al.*, 2013; Mori *et al.*, 2015; Monteiro *et al.*, 2017). Therefore, the novelty in incorporating different remineralising agents into the whitening cycle will provide a better understanding on how much, or little, remineralising agents protect whitened enamel subjected to cyclic erosion and staining, and if different HP pH values play a role in producing better or worse enamel responses to such dietary challenges. As explained in Chapter 4, Nescafe© coffee and 0.3% citric acid were used to stain and erode whitened/remineralised enamel, respectively. Enamel roughness, hardness, mineral composition, and surface morphology were assessed to understand the effects of dietary erosion on whitened/remineralised enamel. Colour measurements were taken to evaluate the degree of enamel stain uptake after dietary staining cycles. This was done in an effort to help achieve maximum whitening results, keep patients informed of the impact of dietary stains on their whitening treatments, and advise on the effectiveness of applying CPP-ACP or 15.5% nHA during the whitening cycle to maintain whitening results for longer durations.

7.8.1 Enamel roughness (Ra)

Dietary erosion leads to a rougher enamel surface (Barac *et al.*, 2015) and whitened enamel, in particular, is more vulnerable to dietary erosion than un-whitened enamel (Attin *et al.*, 2003; Yeh *et al.*, 2005). For that reason, the novelty in testing the effects of integrating remineralising agents into the whitening process using different pH values of HP may be clinically beneficial in understanding the degree of protection these agents might provide against erosive attacks by dietary acids.

Current results revealed that enamel whitened using 6% HP with pHs 5, 7, and 9 and remineralised using CPP-ACP showed no significant changes in Ra after 15 minutes of erosion as compared to their respective control groups where significant changes in enamel roughness was recorded. These findings illustrate the ability of CPP-ACP to protect whitened enamel against 15 minutes of dietary erosion, by this enabling dentists to provide patients with the best whitening treatment while accounting for expected harmful side effects of commonly consumed erosive beverages. Topographical AFM images corresponded to Ra values obtained by clearly demonstrating flat smooth enamel surfaces at BL and after the first erosion cycle, then irregular enamel surfaces after the second and third erosion cycles. This is in line with

previously published studies revealing the effectiveness of CPP-ACP in minimising the harmful effects erosive products have on enamel roughness values through the precipitation of apatite crystals; by this reducing the depth of erosion cavities in treated enamel surfaces (Poggio *et al.*, 2013; Ceci *et al.*, 2015). The application of CPP-ACP significantly reduced enamel damage following cyclic erosion compared to a paste with the same formulation but without CPP-ACP (Ranjitkar *et al.*, 2009). The remineralisation process by CPP-ACP is likely caused by mineral deposition in surface irregularities as opposed to crystal regrowth.

Un-whitened enamel, on the other hand, showed no significant changes in Ra after the first erosion cycle in the un-remineralised group as well as in enamel treated with CPP-ACP. Their topography corresponded to their recorded Ra, revealing surface irregularities only after the second and third erosion cycles. As previously mentioned, whitened enamel is more vulnerable to erosive attacks in comparison to un-whitened enamel (Attin *et al.*, 2003; Yeh *et al.*, 2005), which explains the added benefit CPP-ACP had on whitened enamel in preventing the harmful effects of erosion after the first erosion cycle. This could be attributed to the higher tendency CPP-ACP nano-complexes to form over a pH range between 5 and 9 (Divyapriya *et al.*, 2016). This means that the efficacy of CPP-ACP as a remineralisation agent peaks in that pH range. Furthermore, surface irregularities caused by whitening could lead to greater penetration of CPP-ACP; by this enhancing its ability to remineralise subsurface lesions and with the aid of its relatively low carbonate environment, it creates an enamel surface with improved crystallinity and lower levels of microstrain in comparison to sound enamel, providing a more resistant enamel structure to erosive attacks as seen in current results (Iijima *et al.*, 2004; Divyapriya *et al.*, 2016).

With regards to enamel whitened using pH5 HP and remineralised using nHA, there were no significant changes in Ra values after the first, second, and third erosion cycles. This is in line with previously published studies indicating the significant ability of nHA based remineralising agents in preventing enamel erosion by the development of a new layer of nHA crystals insensitive to dissolution by acidic challenges (Shetty *et al.*, 2014; Singh *et al.*, 2017). In contrast, enamel whitened using 7.5% HP and remineralised using 10% nHA was reported to exhibit a statistically significant increase in Ra after cyclic erosion (Santos *et al.*, 2016). It is worth noting, however, that in this study enamel treated with nHA was significantly rougher at BL with great variability in recorded Ra in comparison to Ra values obtained in enamel treated with CPP-ACP and control enamel. Therefore, the insignificant differences noted could also be attributed to the great variability in Ra values at BL and after each erosion cycle thereafter. Enamel whitened using pH9 HP and remineralised using nHA, on the other hand, revealed a significant increase in Ra after the second erosion cycle followed by no significant change in

enamel roughness after the third erosion cycle in comparison to BL values. In contrast, enamel whitened using pH7 HP and remineralised using nHA revealed no changes in enamel roughness after the first and second erosion cycles followed by a significant decrease in Ra values after the third erosion cycle. Studies investigating the combined effects of HP pH and nHA on enamel's susceptibility to dietary erosion are scarce and according to current results no clear relationship could be identified. Inconsistent Ra results are likely caused by the accumulation of nHA on the treated enamel surface, forming aggregates of nano-crystals which were sporadically distributed, creating an uneven and irregular hydroxyapatite layer on top of the treated enamel surface (Huang *et al.*, 2009). Therefore, Ra results in enamel treated with nHA must be viewed with caution as Ra readings might be of the nHA layer rather than of enamel remineralised with nHA.

7.8.2 Enamel Vickers hardness (HV)

Current results showed that whitened enamel samples were more vulnerable to dietary erosion than un-whitened enamel which is in line with previously published studies (Attin *et al.*, 2003; Yeh *et al.*, 2005). Enamel treated with PBS and not mineralised, revealed no significant changes in HV after the first and second erosion cycles then a significant drop in HV values occurred after the third erosion cycle. In addition, CPP-ACP treated un-whitened enamel showed no significant changes in HV after the first erosion cycle in comparison to BL. Enamel Vickers hardness significantly dropped, however, after the first erosion cycle in samples whitened using pH5 HP whether they were remineralised with CPP-ACP or not. According to Carvalho *et al.*, hardness values in enamel treated with CPP-ACP significantly decreased after being subjected to cyclic erosion (Carvalho *et al.*, 2013). Both treated and control enamel groups had significantly lower hardness values post erosion, similar to findings in this current study. In contrast, others claim that CPP-ACP was effective in preventing the initial demineralisation process which occurs immediately following the exposure to an acidic challenge; visible as hardness reduction in the enamel superficial layer (Fernandes *et al.*, 2019). This is also in accordance with current results, as enamel whitened using pH7 HP and remineralised using CPP-ACP showed no significant changes in hardness values after the first erosion cycle. However, enamel whitened using pH7 HP and not remineralised showed no statistically significant changes in HV after all erosion cycles in comparison to BL. The lack of any statistically significant differences is possibly caused by the great variability in HV values recorded after each erosion cycle. It could also be attributed to minor quantities of alkaline salts present on the enamel surface from the neutral HP solution, rather than from the CPP-ACP agent, which was reported to evenly adhere to treated enamel surfaces forming a protective layer against future erosive attacks (Sun *et al.*, 2011). Enamel treated using neutral whitening agents showed no significant structural changes following an erosive challenge (Pretty *et al.*, 2005). In contrast, enamel whitened using a neutral 35% HP was reported to exhibit a significant reduction in microhardness values after cyclic erosion (Zanet *et al.*, 2011). Controversy exists in regard to the impact of neutral whitening agents in inhibiting or minimising damaging effects of erosive beverages, and whether the application of remineralising agents such as CPP-ACP aid in protecting whitened and unwhitened enamel against dental erosion. In the absence of fluoride, CPP-ACP has a limited capacity to inhibit harmful erosive effects and its remineralisation capability is also limited (Kim *et al.*, 2011). However, according to current results the effectiveness of CPP-ACP in preventing or delaying enamel microhardness changes after cyclic erosion varied according to the whitening agent pH used. Enamel whitened using pH9 HP and not remineralised showed a significant reduction in

HV after all erosion cycles in comparison to BL values, while the CPP-ACP treated enamel, on the other hand, revealed a significant increase in enamel hardness after the first erosion cycle, no significant change after the second erosion cycle, then a significant decrease after the third erosion cycle. This is attributed to the greater damage pH9 HP inflict on the treated enamel surface which could allow CPP-ACP molecules to deeply penetrate, as previously explained, resulting in a possibly more resistant enamel subsurface to subsequent erosion cycles (Iijima *et al.*, 2004; Divyapriya *et al.*, 2016). This is clinically important, as benefits from combining alkaline HP and CPP-ACP in preventing significant changes to enamel hardness post dietary erosion will aid dentists in their decision making when prescribing whitening treatments and help minimise harmful side effects of erosive beverages commonly consumed by the public on whitened enamel surfaces.

The nHA treated enamel showed no significant changes in HV after all erosion cycles in all treatment groups tested. This is in line with results published by Min *et al.*, reporting the effectiveness of nHA in inhibiting enamel hardness reductions following cyclic erosion (Min *et al.*, 2011). The precipitation of nHA crystals were reported to form a layer insensitive to dissolution by acidic challenges (Roveri *et al.*, 2009; Shetty *et al.*, 2014; Singh *et al.*, 2017). For that reason, although the remineralising agent formed an irregular layer on treated enamel surfaces leading to a significant decrease in enamel HV values as mentioned in section 6.7.2, current results showed that nHA is an effective remineralising agent in preventing significant changes in enamel HV after cyclic erosion.

7.8.3 Enamel mineral composition

Repeated exposure to HP and dietary acids was reported to negatively affect enamel hydroxyapatite crystals, through dissolving calcium ions and leaching off other elemental components (de Araujo *et al.*, 2013). According to current results, however, there were no significant differences in Ca, P, and C atomic % between remineralised and control enamel groups post erosion. This is in accordance to previously published literature, reporting no significant changes in enamel Ca and P after being subjected to four 20-minute erosion cycles, repeated for three days (Cheun *et al.*, 2016). Although a slight reduction in Ca atomic % was noted across majority of control groups in comparison to remineralised groups in this study, differences were not significant enough; possibly attributed to the fact that two samples were mapped in each treatment group, which might not be enough to detect any significant remineralising effects by CPP-ACP and nHA similar to what has been reported in previously published research (Hegde and Moany, 2012; Bajaj *et al.*, 2016). It is worth noting however, that these studies tested the restorative capacity of remineralising agents on dental enamel after cyclic erosion as opposed to their ability to prevent/minimise erosive damage in whitened enamel.

The remineralisation process carried out by artificial saliva in between erosion cycles have restored eroded enamel surfaces, which explain the absence of statistically significant differences between test groups (Amaechi and Higham, 2001b; Amaechi and Higham, 2001a; Wang *et al.*, 2011). Storing enamel in AS following exposure to an acid challenge significantly increase mineral gain in eroded enamel, according to readings obtained using contact microradiographs (Kielbassa *et al.*, 2001).

7.8.4 Enamel morphology

According to current results treatment with nHA caused an irregular surface of accumulated nHA crystals which were unaffected by dietary erosion cycles. The nHA crystals aggregate into microclusters, forming an apatite layer on treated enamel surfaces (Swarup and Rao, 2012). In addition, nHA was reported to form a homogeneous apatite coating, insensitive to dissolution by acidic challenges, covering the prismatic and inter-prismatic enamel structures (Roveri *et al.*, 2009; Shetty *et al.*, 2014; Singh *et al.*, 2017). Rinsing enamel with 5% nHA solution was reported to cause the formation of an uneven film of accumulated nHA crystals (Nobre *et al.*, 2020). This was attributed to the electrostatic forces between nano-particles leading to the formation of nano-agglumerates. In contrast, Kensche *et al.* reported that the application of an agent containing HA microclusters followed by dietary erosion caused no significant changes to enamel morphology (Kensche *et al.*, 2016). It is worth noting, however, that the application of the remineralising agent was for one minute and the erosion cycle was performed for 2 minutes which is a significantly shorter duration than that followed in this study.

Enamel whitened using pH5 and pH7 HP and remineralised using CPP-ACP, on the other hand, showed irregular enamel surfaces post erosion, similar to that expressed by their respective controls. The application of CPP-ACP on enamel treated with pH9 HP, however, was effective in minimising microstructural changes after dietary erosion in comparison to other treatment groups. Results reported by Poggio *et al.*, corroborate current findings as they have noted the effectiveness of CPP-ACP in protecting the treated enamel surface by covering enamel prismatic and interprismatic structures, forming a smooth resistant layer to erosive attacks (Poggio *et al.*, 2013). The application of CPP-ACP did not only prevent surface damage after whitening using pH9 HP as mentioned in section 6.7.5, it was additionally effective in minimising microstructural damage after exposure to dietary erosion. Although the effectiveness of CPP-ACP in preventing dental erosion was previously reported in the literature (Manton *et al.*, 2010; Ferrazzano *et al.*, 2012), its significant impact in protecting enamel microstructure against erosive damage after whitening using an alkaline HP hasn't been addressed.

In summary, nHA treated enamel showed a greater degree of surface deformation after dietary erosion in comparison to other treatment groups, while CPP-ACP was effective in minimising microstructural changes following cyclic erosion in samples whitened using pH9 HP.

7.8.5 Enamel colour

In an attempt to reduce the susceptibility of enamel to dietary staining post-whitening, the application of remineralising agents in combination with different pH values of HP was investigated. Current results have shown that treatment with 15.5% nHA caused an irregular enamel surface by the aggregation and clustering of nano-particles and dietary stain up-take in enamel treated with nHA was significantly greater in comparison to enamel treated with CPP-ACP and control samples. This is in agreement with previous research, revealing that nHA did not prevent stain uptake in whitened enamel samples, possibly caused by its low solubility and nonresorbable nature (Rezvani *et al.*, 2015). The application of remineralising agents did not prevent dietary stain uptake in enamel whitened using pH5 HP (Figure 136). A significant reduction in lightness and increase in a* values towards the red end on the spectrum were recorded after the first five minutes of dietary staining in all three treatment groups: CPP-ACP, nHA, and the control. This is in line with previously published research reporting the ineffectiveness of CPP-ACP in reducing stain uptake in whitened enamel, which was attributed to its limited ability in overcoming the surface damage caused by the whitening agent, and hence, reducing the level of stain uptake (Kim *et al.*, 2011; Alaghemand *et al.*, 2015; Monteiro *et al.*, 2017). In addition, it has been claimed that the artificial saliva storage medium has the potential to protect and restore whitened enamel to a possibly similar level as that achieved by CPP-ACP, which might also explain the similar L*, a*, and b* values recorded in this current study in both the CPP-ACP and control groups (Monteiro *et al.*, 2017). Although significant changes occurred in the L* and a* parameters, there were no significant changes in b* values along the yellow-blue spectrum in control enamel samples and enamel treated with CPP-ACP, while nHA treated enamel showed a significant increase in b* values indicating increased enamel yellowness after the first staining cycle in day1. Whitened and remineralised enamel samples using CPP-ACP showed a significant stain uptake ($\Delta E > 2$) after dietary staining with tea (Singh *et al.*, 2010). Authors additionally recorded, similar to current results, no significant changes in b* values along the yellow-blue spectrum in whitened and remineralised enamel after staining. Others reported, however, that CPP-ACP treated enamel showed significantly lower lightness values in comparison to its respective control which was explained by the irregular deposition of calcium and phosphate ions by the remineralising agent which might have created a more susceptible enamel surface to stain uptake (Públio *et al.*, 2013). Similar findings were recorded in this study in enamel whitened using pHs 7 and 9 and remineralised with CPP-ACP and nHA, showing a significant reduction in L* values and a significant increase in b* values towards the yellow end of the spectrum at an earlier stage in comparison to their respective control groups. In regards to un-whitened enamel, on the other hand, lightness

reduction occurred first in enamel treated with nHA, followed by the control group, then finally significant reductions occurred in the CPP-ACP group in day 2 following the third staining cycle. Additionally, enamel treated with CPP-ACP showed less changes in a* values along the red-green spectrum in comparison to other treatment groups and no significant changes in b* values along the yellow-blue spectrum following exposure to all staining cycles. Enamel treated with 10% nHA showed a significant increase in stain uptake ($\Delta E > 2$) following immersion in coffee (Ajami *et al.*, 2016). The application of nHA was reported to cause the formation of an uneven film of nHA agglomerates creating an irregular enamel surface more susceptible to stain uptake and retention which could explain current results (Shannon *et al.*, 1993; Pinto *et al.*, 2004; Tredwin *et al.*, 2006; Huang *et al.*, 2009; Eva *et al.*, 2013; Hassan *et al.*, 2016; Nobre *et al.*, 2020).

In summary, results showed that CPP-ACP had no significant effect in reducing the degree of dietary stain uptake post whitening using pH5 HP, while treatment with nHA caused a greater stain uptake in comparison to other treatment groups. In fact, applying remineralising agents to enamel whitened using pH7 and pH9 HP have led to a more rapid stain uptake in comparison to their respective control groups. Since the main goal of whitening treatments is to whiten dental enamel and maintain results for longer durations, CPP-ACP and nHA should not be applied on enamel whitened using neutral or alkaline HP based on current results. Additionally, remineralising agents were ineffective in preventing dietary staining in enamel whitened using acidic HP and the application of nHA caused a greater stain uptake in comparison to other treatment groups. On the other hand, the application of CPP-ACP could be clinically beneficial in reducing stain uptake in un-whitened enamel, which could potentially eliminate the need for future whitening treatments for some patients.

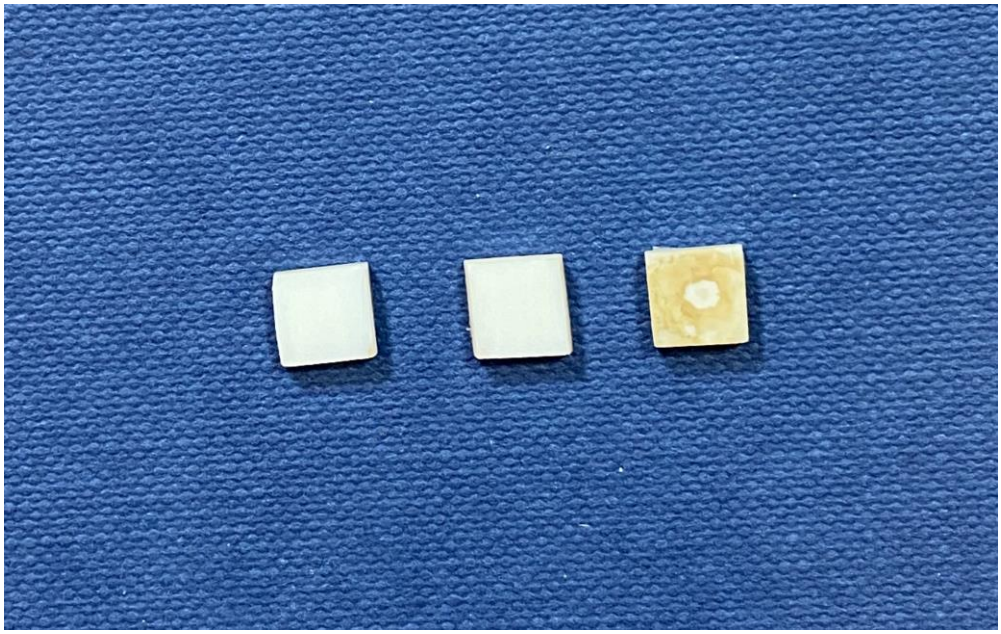


Figure 136 Three enamel samples whitened using pH5 HP and not remineralised (left), remineralised using CPP-ACP (middle), and remineralised using nHA (right) then subjected to staining cycles for three consecutive days.

7.9 Conclusion

After simulated dietary erosion there were no significant differences in enamel mineral composition between all test groups and in comparison to their respective controls. The use of CPP-ACP proved to be beneficial in reducing stain uptake only in un-whitened enamel. In fact, the application of CPP-ACP caused a greater stain uptake in enamel whitened using pH7 and pH9 HP in comparison to their respective controls. The remineralising agent also proved to be effective in preventing significant reductions in enamel hardness after exposure to two erosion cycles in samples whitened using pH9 HP. It also prevented significant changes in roughness values in all whitened enamel samples after 15 minutes of erosion irrespective of the HP pH used. The application of CPP-ACP was additionally beneficial in preventing microstructural changes following cyclic erosion in samples whitened using pH9 HP. The application of 15.5% nHA was effective in protecting enamel against hardness reduction following dietary erosion. It caused, however, a significantly rougher enamel surface and as a consequence enamel treated with nHA exhibited a significantly greater stain uptake following staining cycles. Therefore, the application of 15.5% nHA should not be incorporated into the whitening treatment with the purpose of preventing enamel damage following dietary staining and erosion. The application of CPP-ACP, on the other hand, was effective in preventing erosive roughness and hardness changes in whitened enamel and protected unwhitened enamel against dietary staining.

Chapter 8. General discussion, conclusions, recommendations, and potential future work

8.1 General discussion

Dental whitening is an effective, minimally invasive, and safe treatment for improving patients' smile aesthetics. The chemistry of enamel whitening starts by targeting chromophores which are naturally occurring organic compounds/molecules in enamel and have free extended chains of single and double bonds. Improvement in colour occurs as bonds linking chromophores to enamel are broken through the process of oxidation (Joiner, 2006). HP is the most commonly used whitening agent in dentistry (Bizhang *et al.*, 2017), and in an effort to maximise its whitening efficacy while reducing its negative surface and mechanical side effects, researchers have tested different whitening agent concentrations and pH values on dental enamel. Controversy exists within the published literature around the effectiveness of different HP concentrations and pH variations with some studies reporting significant differences between enamel groups treated with different concentrations/pH values of HP based whitening agents (Moraes *et al.*, 2006; Azrak *et al.*, 2010; Sun *et al.*, 2011; El Halim, 2012; Sa *et al.*, 2012a; Eva *et al.*, 2013; Trentino *et al.*, 2015; Furlan *et al.*, 2017), while others reported no statistical differences between test groups (Cadenaro *et al.*, 2008; De Geus *et al.*, 2018; Jurema *et al.*, 2018). To date, published *in-vitro* investigations concerning the impact of HP concentration and pH on enamel roughness, hardness, colour change, mineral composition, and surface morphology are limited, and results from these studies are difficult to compare as the majority use commercially available products which contain various additives; possibly affecting the accuracy and reliability of any conclusions drawn (Sa *et al.*, 2012a; Trentino *et al.*, 2015; Cvikl *et al.*, 2016).

Remineralising agents play an important role in restoring enamel to pre-whitened conditions and in stabilising the ionic levels of calcium and phosphate (Bayrak *et al.*, 2009). Previously published whitening studies have tested the effects different remineralisation agents have on whitened enamel by either mixing it with the whitening agent, or applying it before or after the whitening treatment (Borges *et al.*, 2011b; de Vasconcelos *et al.*, 2012; Alkhtib *et al.*, 2013; Po and Wilson, 2014). Potential benefits from incorporating remineralising agents into the whitening protocol have not been fully explored and studied. The repeated application of remineralising agents after each whitening cycle could maximise their regenerative potential as opposed to a single application before or after whitening as undertaken in many published studies (Borges *et al.*, 2011b; de Vasconcelos *et al.*, 2012; Alkhtib *et al.*, 2013; Po and Wilson, 2014).

Another important clinical consideration is the ability of remineralising agents to extend the longevity of the whitening effect upon the treated enamel by minimising post whitening staining and erosion caused by dietary products regularly consumed by the general public. To date, research mostly concentrates on the remineralising abilities of various products on eroded enamel rather than studying their ability to prevent dietary erosion in whitened enamel (Hegde and Moany, 2012; Bajaj *et al.*, 2016). In addition, many studies investigating the susceptibility of whitened/remineralised enamel to staining, apply the remineralising agents following whitening treatments (Singh *et al.*, 2010; Públio *et al.*, 2013; Mori *et al.*, 2015; Monteiro *et al.*, 2017). Therefore, the novelty in incorporating different remineralising agents into the whitening cycle will provide a better understating on how much, or little, remineralising agents protect whitened enamel subjected to cyclic erosion and staining, and if different HP pH values play a role in producing better or worse enamel responses to such dietary challenges.

The aforementioned gap in knowledge provided an opportunity to structure a novel approach to test the effects of different combinations of HP concentrations and pH values on bovine enamel roughness, hardness, colour change, mineral composition, and surface morphology. Additionally, the novelty in incorporating remineralising agents into the whitening protocol will help clinicians and scientists develop a whitening regimen using combinations of whitening agent concentrations/pH values and remineralising agents that maximises enamel colour change while minimising structural and mechanical damage commonly caused by whitening treatments. The current research additionally acknowledges the clinical importance of the longevity of whitening results which is commonly affected by dietary staining and erosion (Attin *et al.*, 2003; Yeh *et al.*, 2005; Singh *et al.*, 2010). Therefore, whitened/remineralised enamel samples were subjected to dietary staining and erosion cycles in an effort to assess if different whitening/remineralisation treatments play a role in producing better or worse enamel responses to such challenges.

As previously described, bovine enamel is histochemically and anatomically similar to human enamel, and proved to be a suitable alternative according to previously published *in-vitro* whitening studies (Nakamichi *et al.*, 1983; Al-Salehi *et al.*, 2007; Borges *et al.*, 2015; Mundra *et al.*, 2015; Soares *et al.*, 2016a; Jurema *et al.*, 2018). Furthermore, in an effort to model the oral environment, whitened enamel samples were stored in artificial saliva at 37°C and refreshed every other day (Amaechi and Higham, 2001b; Amaechi and Higham, 2001a; Wang *et al.*, 2011).

Concentrations of HP solutions tested started from 6%; being the maximum allowed HP concentration to be used for dental whitening in the UK (GDC, 2011), up to 40% HP which is a concentration present in commercially available whitening products (Acuña *et al.*, 2019) and

excessive application of such high concentrations of HP was reported to increase the risk of irreversible enamel damage (Lewinstein *et al.*, 2004; Jiang *et al.*, 2008). An intermediate concentration value of 20% HP has been additionally tested to assess the effects of gradual whitening agent concentration elevation on bovine enamel surface quality and mechanical properties. Each HP concentration was tested and divided into three pH values representing an acidic (pH5), neutral (pH7), and alkaline (pH9) HP to investigate the effects of whitening agent pH upon the whitening efficacy of low, medium, and high HP concentrations. The treatment duration of 2 hours/ day for 10 days was prescribed in this current study in line with tray-based whitening protocols previously published ranging between 2-4 hours, daily for 10-14 days (Stokes *et al.*, 1992; Kihn, 2007; Mondelli *et al.*, 2009). Additionally, and according to current pilot results, HP concentrations and pH values selected for Phase 1 have proven to be relatively stable for the 2-hour whitening cycle, which would in turn ensure experimental consistency.

The remineralising agents of choice in this current study were CPP-ACP and nHA, which were applied after each whitening cycle in an effort to protect enamel against harmful structural and surface damage caused by the whitening treatment and by dietary erosion and staining thereafter. As mentioned in previous chapters, nHA is one of the most biocompatible and bioactive materials due to its similarity to dental apatite and its potential to restore dental enamel (Kim *et al.*, 2007; Huang *et al.*, 2010; Juntavee *et al.*, 2018). The concentration and size of nHA crystals chosen in this study were 15.5% and <50nm respectively, which are in line with previously tested nHA concentrations ranging from 1% to 15% and reported apatite crystal size ranging from 20-40nm (Kim *et al.*, 2007; Huang *et al.*, 2009; Swarup and Rao, 2012). Since the nHA paste used was not a commercially available product, the application time was set to follow the same application duration recommended for GC Tooth Mousse (Recaldent™). The CPP-ACP based product applied is a popular remineralising agent recommended for clinical use to restore and remineralise dental enamel (Asokan *et al.*, 2019). Therefore, it is considered as a positive control to the nHA experimental product and differences in their impact on whitened enamel colour, Vickers hardness, roughness average, mineral composition, and surface quality will be discussed below.

Following whitening/remineralisation, treated enamel samples were subjected to 0.3% citric acid with a pH value of 3.8. Citric acid is the most commonly found dietary acid in natural and commercial products, such as orange juice, at a concentration and pH value of approximately 0.3% and 3.8, respectively (Hughes *et al.*, 2000; Austin *et al.*, 2010; Shellis *et al.*, 2011). During the erosion cycle the solution was agitated at 60 rpm to simulate erosive challenges in the oral environment which are unlikely to be static (Mullan, 2018). The duration of each erosion cycle was set to 15 minutes as it has been reported in the literature that early erosive changes in terms

of enamel roughness and hardness occur after 15 minutes (Meurman and Frank, 1991; Mullan, 2018). Therefore, three erosion cycles were undertaken to assess erosive changes in enamel following the exposure to a commonly consumed dietary acid.

Since coffee is a popular beverage, it was selected for the cyclic dietary staining (Mussatto *et al.*, 2011). The beverage was prepared in accordance to manufacturer's instructions and allowed to cool to 60°C which is the mean preferred temperature for hot beverages (Lee and O'Mahony, 2002; Brown and Diller, 2008). Again, the solution was agitated at 60 rpm to resemble oral environment conditions and each staining cycle was set to 15 minutes per day which is in line with the mean consumption time for a cup of coffee (Ertas *et al.*, 2006).

Results in this current research indicate that as HP pH decreases, the overall colour change (ΔE) increases, which was independent of HP concentration used. Literature reporting the whitening effects different HP pH values have on enamel is considerably limited and contradictory, as some have claimed no colour differences were noted after treatment using acidic, neutral, and alkaline HP (Acuña *et al.*, 2019; Balladares *et al.*, 2019), while others report a slight increase in the overall colour change by acidic HP in comparison to alkaline HP (Sun *et al.*, 2011). In addition, claims have also been made on the greater whitening effect alkaline HP possess on treated enamel in comparison to acidic and neutral HP (Xu *et al.*, 2011).

In this study, the overall colour change in treated enamel was directly proportional to the HP concentration used, and increasing the HP concentration produced a greater enamel whitening effect post treatment. This observation concurs with reported literature, illustrating a strong link between whitening agent concentration and the resultant enamel colour change (Kawamoto and Tsujimoto, 2004; Fearon, 2007; Borges *et al.*, 2015). Although a gradual increase in ΔE has been observed in this current study as a consequence to elevating the HP concentration, values did not significantly differ upon increasing the concentration from 6% to 20%. The additional increase, on the other hand, to 40% significantly enhanced the overall whitening effect which was only recorded in enamel whitened using pH5 and pH7 HP solutions. This means that at higher concentrations, acidic and neutral HP are more likely to produce a greater whitening effect than alkaline solutions.

Incorporating CPP-ACP and nHA into the whitening cycle did not significantly affect the resultant enamel colour in all treatment groups except in samples whitened using pH5 HP and remineralised using CPP-ACP. A statistically significant greater increase in L* values was recorded which contributed to a significantly greater ΔE in samples whitened using an acidic 6% HP and remineralised using CPP-ACP in comparison to those remineralised using nHA and control enamel samples. Treatment with CPP-ACP caused a significant increase in L* values in enamel whitened using an acidic 20% CP (Shirani *et al.*, 2015). The application of CPP-ACP

was reported to effectively restore rough demineralised enamel, by this enhancing its translucency and lustre (Manton *et al.*, 2008). Therefore, treatment with an acidic HP; reported to produce a frosted glass effect which gives an illusion of a lighter surface (Xu *et al.*, 2011) in combination with CPP-ACP claimed to give enamel a "lighter than normal appearance" could explain the significantly lighter surface in enamel whitened using pH5 HP and remineralised using CPP-ACP in comparison to other treatment groups (Shirani *et al.*, 2015).

The susceptibility of whitened/remineralised enamel to dietary staining has been additionally tested in this study, to not only reveal the impact remineralising agents, HP pH, and HP concentration have on enamel whitening outcome, but to evaluate the degree of protection these combinations provide against dietary stain uptake post whitening. Current results indicate that CPP-ACP had no significant effect in reducing the degree of dietary stain uptake post whitening using pH5 HP. In fact, applying remineralising agents to enamel whitened using pH7 and pH9 HP have led to a more rapid stain uptake in comparison to their respective control groups. Previously published studies have also reported the ineffectiveness of CPP-ACP in reducing stain uptake, which was attributed to its limited ability in overcoming the surface damage caused by the whitening agent, and hence, reducing the degree of staining (Kim *et al.*, 2011; Alaghemand *et al.*, 2015; Monteiro *et al.*, 2017). Furthermore, nHA treated enamel produced a significantly rougher surface in all treatment groups, lower degree of colour change post whitening in comparison to other groups, and greatest stain uptake after being exposed to dietary staining cycles. The use of 15.5% nHA in this study caused nano-crystals to visibly cluster on the whitened enamel surface according to SEM images (Figure 77, 83, 89, 95), forming thin fragile irregular overlapping layers of nanocrystals which contributed to the increased enamel roughness, making treated enamel surfaces more susceptible to stain uptake (Watts and Addy, 2001).

After treatment with 6% HP, a significant increase in enamel Ra only appeared in samples whitened with alkaline solutions and increased in severity with the increase in HP concentration. Acidic HP caused a significant increase in enamel roughness after treatment with 20% HP. There were no significant changes in enamel Ra, however, after treatment with 40% HP due to the softening of the enamel surface after being exposed to a highly erosive solution. This was confirmed by hardness results, revealing significantly lower hardness values in enamel treated with an acidic 40% HP compared to other concentrations tested. On the other hand, treatment with neutral HP showed the most favourable response to increasing the solution concentration, revealing statistically significant changes in enamel Ra only after treatment using 40% HP. Current results have additionally revealed that changes in whitened enamel roughness were not prevented by the application of remineralising agents. In fact, the

application of nHA created a rougher enamel surface, irrespective of the HP pH tested. This is possibly caused by the aggregation of nano-crystals on the whitened enamel surface (Swarup and Rao, 2012; Rezvani *et al.*, 2015) by the continuous application of nHA paste after each whitening cycle, creating an irregular fragile apatite layer.

In all whitened enamel groups, CPP-ACP provided a protection against 15 minutes of erosion using 0.3% citric acid which is equivalent to being exposed to orange juice. This was shown by the absence of any significant changes in enamel Ra after the first erosion cycle as opposed to their respective control groups. This is in line with previously published studies revealing the effectiveness of CPP-ACP in minimising the harmful effect erosive products have on enamel roughness values (Poggio *et al.*, 2013; Ceci *et al.*, 2015). Conversely, inconsistent Ra readings were noted in the nHA group post erosion, possibly caused by the accumulation of nHA on the treated enamel surface forming aggregates of nano-crystals. These crystals were sporadically distributed, creating an uneven and irregular hydroxyapatite layer on top of the treated enamel surface as described earlier (Huang *et al.*, 2009). Therefore, Ra results in the nHA group must be viewed with caution as Ra readings might be of the nHA layer rather than of enamel remineralised with nHA crystals.

According to results, all whitening treatments caused a significant reduction in enamel hardness. After treatment with 6% and 20% HP, enamel hardness among pH groups was not significantly different, indicating that at a constant HP concentration pH plays no role in minimising the severity of micro-hardness reductions. This concurs with previously published research, revealing no significant differences between enamel samples treated using HP with pHs 5, 7, and 8.4 under the same concentration value (Jurema *et al.*, 2018). This, along with current results, demonstrate the complex interaction between both HP concentration and pH. Hardness readings have additionally shown that increasing the concentration of an acidic (pH5) or an alkaline (pH9) HP solution will have a greater damaging effect on enamel micro-hardness levels in comparison to a neutral (pH7) HP solution. Treatment with 6%, 20%, and 40% HP solutions with a pH value of 7 caused the same degree of reduction in HV (23-24%).

There is a direct relationship between enamel micro-hardness values and mineral composition (Pizani *et al.*, 2015). However, although changes in enamel hardness were significant after whitening treatments current results showed no significant changes in enamel Ca, C, and P after whitening using 6% and 20% HP with pH values of 5, 7, and 9. The remineralisation effect of the artificial saliva used as a storage medium could have contributed to the absence of any significant changes in enamel minerals (Amaechi and Higham, 2001b; Amaechi and Higham, 2001a; Wang *et al.*, 2011). The atomic % of P, on the other hand, significantly increased in enamel exposed to 40% HP, irrespective of its pH value, in comparison to other concentration

groups. According to Wang *et al.*, EDX quantification of elemental composition should be interpreted with caution, and must be viewed in terms of relative as opposed to absolute amounts (Wang *et al.*, 2019). Furthermore, studies have shown that the greatest disadvantage of EDX quantitative elemental analysis, is its low energy resolution which could potentially lead to spectrum overlapping and misidentification of elements, therefore, caution and attention are required during analysis (Harada and Ikuhara, 2013). The absence of significant changes in Ca, C, and P in enamel treated with 6% and 20% HP with pHs 5, 7, and 9 in this study and in previously published studies (Çakır *et al.*, 2012; Coceska *et al.*, 2016; Ahmed, 2019), indicate that enamel surface mineralisation is not significantly affected by whitening treatments using HP concentrations up to 20%.

Results obtained following whitening treatments were reflected on enamel morphology according to SEM images showing a greater degree of surface damage as HP concentration increased. Enamel whitened using alkaline HP, showed the greatest surface damage in the form of pits, craters, and loss of prismatic/ interprismatic enamel under all HP concentrations tested (Figure 67-69). Enamel treated with acidic HP, on the other hand, started showing erosive damage at 20% HP concentration which became more sever after treatment with 40% HP. The neutral HP, however, did not cause any surface deformation at 6% and 20% concentrations, then loss of prismatic/interprismatic enamel and the development of groves appeared after treatment using neutral 40% HP.

The application of CPP-ACP was ineffective in minimising enamel Vickers hardness reduction post whitening using 6% HP. This is in agreement with a study by Kutuk *et al.*, revealing no significant differences in enamel hardness reductions after whitening using 38% HP followed by the application of CPP-ACP in comparison to samples whitened with no remineralising agent applied (Kutuk *et al.*, 2018). This could be attributed to the low remineralisation capacity of CPP-ACP caused by the artificial saliva storage medium as explained earlier, thus limiting its regenerative benefits. However, CPP-ACP was able to protect enamel whitened using alkaline HP from surface damage by maintaining a smooth flat unaffected surface as compared to the damaged control (Figure 90). The irregular enamel surface following treatment with alkaline HP might have provided a greater space for CPP-ACP to precipitate and cover the prismatic/interprismatic cavities, by this forming a smooth resistant layer to future attacks (Poggio *et al.*, 2013). Results have also shown the effectiveness of CPP-ACP in preventing significant changes in enamel hardness after 15 minutes of erosion in samples treated with neutral HP, and after two 15-minute erosion cycles in enamel treated with alkaline HP. It is possible that the precipitation of alkaline salts, as a result of treatment using neutral and alkaline HP in combination with the alkaline nature of CPP-ACP, had a buffering effect on the citric

acid solution used for erosion, by this minimising its erosive impact while increasing enamel resistance to erosive damage (Khoroushi *et al.*, 2016). The application of CPP-ACP was also effective in producing a smoother enamel surface post whitening using pH9 HP according to SEM images as compared to the control (Figure 78, 84, 90). It did not however, along with nHA, have any added benefit on enamel mineral composition post erosion as compared to control enamel groups. This could be explained by the remineralisation process carried out by artificial saliva in between erosion cycles; possibly restoring eroded enamel minerals to BL levels (Amaechi and Higham, 2001b; Amaechi and Higham, 2001a; Wang *et al.*, 2011).

The application of nHA had a negative impact on enamel hardness values. Indeed, the use of 15.5% nHA contributed to the dramatic decrease in enamel hardness values after whitening in comparison to other treatment groups as the indenter was probably measuring the hardness of the nHA layer rather than the treated enamel surface. The irregular sporadically distributed apatite clusters have resulted in great variability in recorded HV results. The application of 15.5% nHA was, however, effective in protecting enamel against hardness reduction following dietary erosion. It caused, however, a significantly rougher enamel surface and as a consequence enamel treated with nHA exhibited a significantly greater stain uptake following staining cycles.

The current *in-vitro* study was undertaken to assess the effects of high, intermediate, and low HP concentrations with acidic, neutral, and alkaline pH values on bovine enamel. Attempts were made to closely model the oral environment, however, an exact replica of such complex conditions with various confounding factors i.e. oral flora, natural saliva, dietary habits, etc. is extremely difficult to achieve. Additionally, HP solutions were tested in this research as opposed to commercially available whitening agents. Although this might be considered a limitation, HP solutions were used to ensure that any changes recorded in whitened enamel are caused by the HP concentration/pH as opposed to other additives present in the whitening agent posing as confounding variables. This will aid clinicians in adopting an evidence based approach upon prescribing the most effective whitening agent concentration and pH and understating the extent remineralising agents such as CPP-ACP and nHA protect enamel against damages caused by the whitening agent and/or prevent detrimental effects from dietary staining and erosion thereafter. Qualitative and quantitative assessment methods were used to study enamel's response to whitening, remineralisation, in addition to staining and erosion cycles. This provided a more rounded view of changes occurring in enamel following these treatments and helped establish meaningful associations between different results; e.g. the increase in enamel Ra following treatment with nHA contributed to a greater colour change following cyclic dietary staining.

8.2 Conclusion

The present *in-vitro* study investigated the effects of HP concentration and pH on bovine enamel. In addition, the effects of remineralising agents on whitened enamel and its response to dietary staining and erosion were assessed. The study was divided into three main phases:

Phase I: The effects of HP concentration and pH on enamel.

Phase II: The effects of remineralising agents on whitened enamel.

Phase III: The effects of dietary erosion and staining on whitened/remineralised enamel.

Within the limitations of this study the following conclusions for each research phase were drawn:

8.2.1 Phase I: The effects of hydrogen peroxide concentration and pH on enamel

- Whitening using 6% HP with pH values of 5 and 7 resulted in no significant changes in enamel Ra. Treatment with alkaline 6%, 20%, and 40% HP, on the other hand, caused the greatest increase in enamel roughness in comparison to acidic and neutral HP solutions.
- Reductions in enamel micro-hardness (HV) were directly proportional to the concentration of acidic (pH5) and alkaline (pH9) HP, while solutions with a pH value of 7 caused the same degree of reduction in enamel HV (23-24%) at 6%, 20%, and 40% HP concentrations.
- Recorded ΔE values did not significantly differ upon increasing the HP concentration from 6% to 20% while 40% HP caused a significantly greater whitening effect. At each concentration value the whitening effect of HP was inversely proportional to solution pH.
- Alkaline HP caused significant morphological and topographical damage to treated enamel samples according to SEM and AFM respectively; being directly proportional to the HP concentration.

8.2.2 Phase II: The effects of remineralising agents on whitened enamel

- The application of 15.5% nHA significantly reduced enamel HV, increased enamel Ra, resulted in lower overall colour change (ΔE), and significantly damaged enamel microstructure. The use of CPP-ACP, on the other hand, significantly improved the resultant colour in samples whitened using pH5 HP, and prevented morphological damage in samples whitened using pH9 HP.
- The application of 15.5% nHA created a significantly irregular surface in whitened and un-whitened enamel according to SEM images.

- The combined whitening/ remineralisation protocols did not significantly affect enamel mineral composition.

8.2.3 Phase III: The effects of dietary erosion and staining on whitened/remineralised enamel

- The application of 15.5% nHA was effective in protecting enamel against hardness reduction following dietary erosion. It caused, however, a significantly rougher enamel surface and as a consequence enamel treated with nHA exhibited a significantly greater stain uptake following staining cycles.
- The application of CPP-ACP was effective in preventing erosive roughness and hardness changes in whitened enamel and protected unwhitened enamel against dietary staining.

8.3 Recommendations

According to results obtained from this current study the following recommendations have been made:

- Whitening using 6% HP, as it caused a whitening effect similar to that recorded after treatment with 20% HP while causing the least damage to enamel.
- Treating enamel with neutral HP as it produced the least damaging effect with satisfactory colour results in comparison to solutions with other pH values at low, intermediate, and high HP concentrations.
- The incorporation of CPP-ACP into the whitening protocol to prevent morphological damaging effects caused by whitening using alkaline HP.
- The incorporation of CPP-ACP in the whitening protocol to prevent enamel damage caused by dietary erosion post whitening.
- Eliminate the incorporation of 15.5% nHA into the whitening cycle as results have shown its negative impact on treated enamel samples.

8.4 Potential future work

According to results obtained from this current study the following recommendations have been made for future studies:

- Incorporating different concentrations of nHA to whitening protocols, to compare their effects on enamel Ra, HV, colour change, enamel morphology, and mineral composition post treatment.
- Using human saliva as a storage medium to closely model the remineralisation and demineralisation processes occurring in the oral environment.
- Using remineralising agents such as bioactive glass and CPP-ACFP, and dietary staining and erosive solutions such as tea and cola for example to gain a better understanding on the degree dietary options and remineralising agents affect whitened enamel.

Chapter 9. References

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Appendix A. Published IADR abstract orally presented at IADR June 2019 in Vancouver - Canada

IADR Abstract Archives

The Impact of the ph of Hydrogen Peroxide on Enamel Surface Properties

Objectives: Hydrogen peroxide (HP) based whitening products can damage enamel due to their low pH. Consequently, manufacturers have looked to increase the pH. This *in-vitro* study investigates the effect of HP pH on roughness, colour change (△E) and chemical composition of bovine enamel.
Methods: 20wt.% HP was adjusted with either 0.25M NaOH or 0.1M HCl to give pHs of 5,7 and 9. These were used to treat polished bovine enamel samples (6x6mm, n=5 per pH), for 2hours, daily for ten days. A control group was treated with phosphate-buffered saline (pH7.4). After treatment, all groups were stored in artificial saliva (AS) at 37°C. Measurements of enamel roughness and colour were investigated before and after treatment using atomic force microscopy, and spectrophotometry. Mineral loss and qualitative evaluation of treated enamel surfaces were undertaken using energy dispersive x-ray spectroscopy (EDX) and scanning-electron microscopy (SEM), respectively.
Results: As pH values decreased there was a statistically significant increase in △E($P<0.05$), indicating a greater whitening effect, Table1. There was no clear trend found for roughness (Ra); the greatest increase compared to pre-treatment (78%) was found for the pH9 treated samples ($P<0.05$). EDX revealed no statistically significant mineral loss post-treatment for any group. SEM images revealed distinct surface changes with development of craters, loss of inter-prismatic enamel, and formation of grooves on pH9 treated samples. Other groups showed smooth enamel surfaces, with minor mineral deposits in the untreated group, possibly caused by AS.
Conclusions: While there was a decrease in colour change as pH increased, all treated specimens exhibited significantly greater △E than the control-group. All HP-treated specimens showed increased Ra, with greatest changes measured in the alkaline group (pH9) potentially due to irreversible surface damage.

Division: IADR/AADR/CADR General Session
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SESSION INFORMATION
Oral Session
Color and Appearance (Esthetics): Tooth Whitening, Resin Composites
Saturday, 06/22/2019 , 08:00AM - 09:30AM

TABLES

Treatment	Colour change	Ra (nm)	
	ΔE	Before	After
pH 5	11.02	5.6 (1.6)	7.03 (2.2)a
pH 7	9.1	6.4 (3.0)	6.9 (2.3)
pH 9	8.33	6.8 (4.8)	12.1 (44.0)a
Control	3.11	7.6 (2.8)	6.1 (2.1)a

Table.1

IMAGES

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Appendix B. Publication of a review article in Journal of Dentistry in June 2020.

Journal of Dentistry
Available online 29 June 2020, 103423
In Press, Journal Pre-proof

Review article

A Review on Dental Whitening

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Abstract

Objectives

To provide a narrative review on vital dental whitening chemistry, toxicity and safety, vital dental whitening techniques, whitening systems, potential side effects of whitening and cyclic whitening using products with a range of concentrations and pH values. In addition, new developments and recommendations in the field of vital dental whitening will be presented to help clinicians understand the whitening process, its advantages, limitations, and the impact of whitening concentration and pH on enamel providing guidance in tailoring whitening treatments.

Data

Data were gathered using the following keywords: dental whitening, roughness, hardness, sensitivity, hydrogen peroxide, whitening pH, whitening concentration, whitening chemistry, colour, and toxicity.

Sources

An electronic search was performed using PubMed and Scopus databases. Bibliographic material from papers reviewed was then used to find other relevant publications.

Conclusions

The effectiveness of vital dental whitening depends on many factors, such as the concentration/pH of the whitening agent, application duration, chemical additives, and re-mineralising agents used. Developing new whitening products and technologies such as nano-additives and alternative carrier systems is showing promising results, and might prove efficient in maximising whitening benefits by accelerating the whitening reaction and/or minimising expected reversible/irreversible enamel structural damage.

Keywords

dental whitening; hydrogen peroxide; whitening pH; whitening concentration; whitening chemistry