A novel cycling model to investigate the effects of energy and sports drinks on enamel

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Abstract

A novel method to investigate the effects of energy drinks

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Objectives

To develop a novel *in vitro* cycling model which represents a 24-hour dietary cycle and investigate the effects of energy and sports drinks on bovine enamel. To assess if exposure to these drinks results in enamel mineral loss in sound and demineralised enamel.

Methods

A newly designed and novel pH cycling model which simulates the daily dietary cycle with respect to pH fluctuations within the oral cavity was developed using an automated pH cycling robot. The model was used to assess the effects of the Red Bull energy drink, Monster energy drink and Lucozade Sport drink on sound and demineralised enamel samples. Bovine incisors were highly polished and sectioned into 5mm x 4mm samples with acid resistant nail varnish on the margins leaving an exposed central window. Seventy-five (n=75) enamel samples were randomised into 5 groups. Each sample was further divided to create a pair, with one pair remaining sound and the other undergoing artificial demineralisation. Artificial carious lesions were produced for the demineralised samples prior to the start of the cycling study. An automated pH cycling robot was programmed to run the *in vitro* study over 24 hours for 14 consecutive days. The outcome measure of enamel mineral loss was measured using Quantitative Light Fluorescence (QLF) and Transverse Micro Radiography (TMR). Data was collected at baseline (T0) and at the end of the cycling study (T1).

Results

QLF analysis showed mineral loss (ΔF) in all test drink samples, in both the sound and demineralised samples. Sound samples in the Lucozade sport group had the highest mineral loss and in the demineralised samples, Monster energy group had the largest increase in mineral loss at the end of the main cycling study. TMR analysis confirmed

all test drinks resulted in mineral loss (ΔZ) with erosive lesions evident in sound enamel samples and further mineral loss in demineralised samples. In the sound samples, Monster group had the highest mineral loss and for demineralised samples, Red Bull samples had the largest increase in mineral loss. ANOVA with post hoc Tukey Test and ANCOVA with Estimated Means Testing confirmed that demineralisation occurred in all test drinks samples with statistically significant differences for mineral loss when compared with the negative control.

There were no significant differences in mineral loss between the test drinks.

Conclusions

Exposure to Red Bull, Monster and Lucozade Sport results in significant enamel mineral loss in sound bovine teeth and demineralised bovine teeth after 14 days of *in vitro* cycling.

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Chapter 1 Literature Review

1.0 Literature Review

1.1 Background

The consumption of energy and sports drinks has increased significantly in the United Kingdom (U.K.) in recent years with a large proportion of those consuming these drinks being adolescents and young adults (Zucconi *et al.* 2013). Energy drinks are made to supply enough carbohydrates to increase energy as well as increase alertness and offer other physiological benefits (Thomas *et al*. 2016). These drinks however also have adverse effects.

It is important to fully appreciate the terminology with regards to the classification of beverages. Energy drinks are a group of non-alcoholic drinks which contain caffeine and other ingredients to create a stimulating physiological effect (HC821 Science and Technology Committee report 2018). These beverages are different from Sport drinks, which were developed to provide electrolyte and carbohydrate replacement (Higgins *et al.* 2010). Recognition of this distinction between energy and sports drinks is important, as the two groups of beverages have different ingredients and therefore different physiological effects on the consumer.

The potential functional benefits of energy drinks can be linked to their ingredients. Energy drinks are carbonated beverages which contain carbohydrates, electrolytes (calcium, magnesium, sodium, and potassium), and stimulants such as caffeine and taurine (British Soft Drinks Association 2016). Although some of these ingredients are important for body function, namely electrolytes and carbohydrates, the other ingredients can have potentially adverse effects on oral and general health.

Energy drinks are also recognised for their adverse health effects, not only due to the high caffeine content, but also due to their high sugar content and low pH. The high sugar content of energy drinks has implications for oral and general health and has been known to cause dental caries (Moynihan and Kelly 2014), dental erosion (Pinto *et al.* 2013) and obesity (Te Morenga *et al.* 2012).

Despite these reported adverse health effects, energy drinks still remain very popular with athletes, schoolchildren and adolescents. It is reported that amongst British athletes, energy drinks are the most popular supplement (Petroczi *et al.* 2008). This may be due to the potential benefits that these drinks offer, such as boosting energy and increasing alertness (Kumar *et al.* 2015). It is thought that energy drinks can improve stamina as well as performance and this can allow for longer periods of activity or work (Ullrich *et al.* 2015). Due to these perceived benefits, one can see the appeal of energy drinks to a large cohort of the population.

A recent cross-sectional study conducted in the U.K. assessed the oral health-related behaviours reported by elite and professional athletes (Hinds 2019). It was shown that most (94.2%) of the study subjects brushed their teeth twice daily. This study concluded that despite reporting positive oral health-related behaviours, athletes have substantial amounts of oral disease. The consumption of sports nutrition products, which includes energy drinks, was common in 80% of the study subjects. It is thought that irrespective of good oral health practices, due to the consumption of high sugar drinks, consumers are still at high risk of oral disease.

Alarmingly, the consumption of energy drinks amongst adolescents is correlated with engaging in other high-risk behaviours such as drinking alcohol and smoking. In the study by Miller *et al.* (2008), they concluded that their findings suggest that energy drink consumption is closely associated with a problem behaviour syndrome, particularly amongst the Caucasian population (Miller *et al.* 2008). This is a worrying correlation and shows the extension of the potential harmful effects that energy drinks can have on consumers. Perhaps equally, or arguably even more worrying, is the current evidence which relates the harmful effects of energy drinks on children. The European Food Safety Authority (EFSA) have reported evidence that excess consumption of energy drinks by children is linked to a number of negative health effects on physical and mental health. These include sleep disturbances, headaches, tiredness and irritation (EFSA 2015).

It has therefore come as welcome news, that during the writing of this thesis, the U.K. government announced that they will hold further consultations to assess whether to

introduce legislation to ban the sale of energy drinks to those under the age of 16 years old (Government response to the House of Commons Science and Technology Committee report on Energy drinks and children 2019). A number of leading U.K. supermarket retailers have voluntarily introduced a ban of the sale of energy drinks for those under the age of 16 years. This too is welcome news and demonstrates the growing awareness of the negative impact of the consumption of energy drinks in children and adolescents.

There have been several studies that have taken place to evaluate the issues regarding the overall intake of energy drinks and their adverse effects (Higgins *et al.* 2010, Campbell *et al.* 2013). The British Soft Drinks Association (BSDA) Annual Report in 2015 showed that consumption of energy drinks in the UK had increased by 155% from 2006-2014. Furthermore, the data for energy and sports drinks consumption from 2015 to 2019 has shown a year-on-year growth ranging from 1.3% to 6.3%. This substantial rise shows the increasing popularity of energy drinks in recent times (BDSA Annual Report 2021). Energy drinks and carbonated soft drinks account for 6.2% of Total Soft Drinks consumption in the U.K. with the clear majority of this share (86.3%) owing to the sales of energy drinks (British Soft Drinks Annual Report 2021).

The BSDA Annual Report 2021 published sales statistics for energy and sports drinks consumption in the U.K. At the time of writing this thesis, the most up to date statistics for energy drinks consumption in the U.K. showed that 836 million litres of energy and sports drinks were consumed in the U.K. in 2020, at a retail sales value of £2,089,000,000. (BDSA Annual Report 2021). Surprisingly, when compared with the previous year (2019), the statistics for 2020 show a decrease in the year-on-year growth for energy and sports drinks sales in the U.K. It can be proposed that the sales statistics for 2020 are an anomaly when compared to previous years and that the data for 2020 is not truly reflective of the potential sales due to the COVID-19 Pandemic, which may have led to a reduction in retail sales caused by national/regional lockdowns and social mobility restrictions globally.

The retail sales statistics confirm that these drinks are very popular and in recent years, there is a general increase in the consumption of energy and sports drinks in the U.K.

It is therefore imperative that the effects of these on the dentition is investigated in an effort to raise public awareness of their potential adverse results.
The British Soft Drinks Association define Sports drinks as functional drinks which are specifically designed to help athletes and other active people hydrate before, during and after exercise (British Association Soft Drinks 2021). Sports drinks differ from energy drinks in terms of certain ingredients as well as retail marketing strategies. Sports drinks brands are regularly marketed via sports ambassadors and this association and alignment with sport portrays a positive and healthy image for their product (Fairchild *et al*. 2017).

Sports drinks were first created in 1965 by Robert Cade in the United States of America in an effort to address certain physiological and nutritional issues related to sporting events (Burke and Read 1993). This particular beverage was designed for athletes needing replenishment of water as well as carbohydrates and electrolytes lost during prolonged vigorous physical activity, including activities performed in high temperatures and humidity (Healthy Eating Research 2012). Since this first batch of this experimental sports drink, an entire industry has arisen with many major drinks brand having invested in developing their own sports drinks aimed at the mass market (Cohen 2012). The marketing of sports products is a multimillion-pound industry (Heneghan *et al*. 2012) and recent data shows that in the U.K. the core consumers were 15-24 year olds (Mintel 2014).

Despite the perceived benefits of sports drink, these drinks have the potential to be damaging to oral and general health. Sports drinks contain free sugars and acids, and this combination results in these drinks having the potential to cause dental caries and erosion (Noble *et al.* 2011). Most sports drinks have a pH ranging from 3-4 and this low pH is associated with enamel demineralisation (Shaw and Smith 1999). Furthermore, as sports drinks are primarily targeted at athletes and individuals partaking in sporting activities, the potential adverse effects can be further compounded by dehydration of the consumer. Dehydration associated with sporting activities increases the risk of dental erosion, as the buffering capacity of saliva is inhibited as a result of lower salivary flow. The lower salivary flow rate also means that

there is reduced clearance of acids and sugars from the tooth surface, which can affect dental caries and erosion (Noble *et al.* 2011).

It is imperative to ascertain what effects these drinks have when consumed on a regular basis. Therefore, this *in vitro* study has designed a novel cycling model to represent a 24-hour cycle which mirrors the pH fluctuations within the oral environment. By incorporating energy and sports drinks within the cycling protocol, we have been able to ascertain what effects these drinks have on bovine enamel when consumed on a regular basis. This research study will aid and inform further studies assessing the effect of energy drinks on human enamel and pave the way for further studies to be conducted.

1.2 Dental Enamel

Teeth consist of mineralised tissues which surround an inner core of loose connective tissues. Enamel is one of the three mineralised tissues which make up a tooth and covers the outermost surface of the clinical crown. Enamel is the hardest biological tissue in the human body and is highly mineralised. Ameloblasts are the cells which are responsible for the formation of enamel. It is important to appreciate that ameloblasts are lost following the eruption of the tooth into the oral cavity. As such, enamel is unable to renew itself. This means that damage to the enamel is generally irreversible, as the enamel does not have the capability to regenerate and restore itself (Nanci and Ten Cate 2018).

The principal mineral component of enamel is calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and this mineral comprises of approximately 96% of the tissue by weight. The remaining 4% is organic material and water. The inorganic content of enamel is a crystalline calcium phosphate (hydroxyapatite) substituted with carbonate ions, which is also found in bone, calcified cartilage, dentine and cementum. If there are ions, such as strontium, magnesium, lead and fluoride, present during the formation of enamel, then these ions can be incorporated into the crystals. The susceptibility of these crystals to dissolution by acid provides the chemical basis for demineralisation and subsequently, dental caries (Nanci and Ten Cate 2018).

The fundamental organisational units of mammalian enamel are rods and inter-rod enamel (inter-prismatic substance) (Nanci and Ten Cate 2018). These rods (sometimes referred to as Prisms) consist of several million crystallites packed into a long thin rod of 5-6µm in diameter and up to 2.5mm in length (Berkovitz *et al*. 2009). The thickness of enamel varies according to the anatomical site. Enamel is thickest at the occlusal or incisal surface and thinnest at the cervical margins.

The process of enamel formation is known as amelogenesis. Ameloblasts secrete matrix proteins and are responsible for creating and maintaining an extracellular environment favourable to mineral deposition. Amelogenesis is generally subdivided into 3 main functional stages, referred to as the presecretory, secretory and maturation stages. During the presecretory stage, differentiating ameloblasts acquire their phenotype, change polarity, develop an extensive protein synthetic apparatus, and prepare to secrete the organic matrix of enamel. During the secretory stage, ameloblasts elaborate and organise the entire enamel thickness, resulting in the formation of a highly-ordered tissue. Lastly, during the maturation stage, ameloblasts modulate and transport specific ions required for the concurrent accretion of mineral (Nanci and Ten Cate 2018).

It is pertinent to mention that disturbances within the amelogenesis process can have a detrimental effect on the development and formation of enamel. Amelogenesis Imperfecta is a group of inherited defects that cause disruption to the structure and clinical appearance of tooth enamel. It is associated with mutations in five genes (AMELX, ENAM, MMP20, KLK4 and FAM83H) (Gadhia *et al.* 2012). This disorder leads to reduced thickness of enamel, as well as pitting and grooves. The weakened structure of the enamel results in an increased susceptibility to demineralisation and subsequently dental caries. It is therefore important for the purposes of research, to exclude from the study any teeth with signs of developmental disturbances such as amelogenesis imperfecta, as this may lead to issues pertaining to the reliability and validity of the study.

Acid etching of enamel is a common clinical procedure in dentistry. This process involves the use of an acid (typically 37% ortho-phosphoric acid) to condition the

superficial surface of the enamel. It is used in a number of clinical applications such as bonding of restorative materials, use of fissure sealants, and of particular relevance to the primary author of this study, for bonding of orthodontic brackets to the enamel tooth surface. The process of acid etching achieves the desired effects in two stages: firstly, the acid etching removes any superficial plaque and debris, and secondly by increasing the porosity of exposed surface through selective dissolution of crystals, providing a better bonding surface for the respective restorative or adhesive material (Nanci and Ten Cate 2018).

Both human and bovine teeth are extensively used for the purposes of dental research (Mellberg 1992, Shellis *et al.* 2011). Human teeth may be preferred for *in vitro* and *in situ* dental research as this allows for more clinically applicable testing of the study hypothesis. Whilst the majority of *in vitro* testing of dental materials is performed on extracted human teeth (Teruel *et al.* 2015), there remains several limitations to the use of human teeth in dental research purposes.

These limitations include difficulty in obtaining sufficient quantity and with sufficient quality, as they are likely to have been extracted due to extensive dental caries and other diseases (Mellberg 1992). It may be challenging to establish sample homogeneity and to control the source and age of the collected human teeth, leading to larger variations in the outcome measures (Zero 1995). Furthermore, human teeth also have previous exposure to fluoride which may be seen as a disadvantage for experimental research purposes.

In cases where human teeth are not used, other mammal species are used as substitutes for human teeth for dental research. The most common candidate for the replacement of human teeth are bovine teeth (Yassen *et al.* 2011). Bovine teeth have the advantage of offering a larger and flatter enamel surface for testing, whereas human teeth have the limitation of having a smaller and relatively curved surface area (Zero 1995). This is of particular importance in this study, as a flat surface is preferable for testing and the flatter and relatively larger surface provided by a bovine incisor is advantageous for experimental purposes.

Bovine teeth have been the most widely used substitute for human teeth in dental studies and their use has dramatically increased over the last 30 years (Yassen *et al.* 2011). Despite this increased use, it is important to note that the chemistry and structure of bovine teeth is not identical to human teeth (Teruel *et al.* 2015). In previous studies investigating the chemical composition of bovine enamel, it has been found that acid etching of bovine enamel results in the formation of a rougher surface and the hydroxyapatite crystals are oval shaped and narrow, in contrast to the round shape found in human enamel (Nakamichi *et al.* 1983).

Numerous studies have been conducted to compare dental caries directly between human and bovine teeth. (Featherstone and Mellberg 1981, Teranaka *et al.* 1986, Edmunds *et al.* 1988, Kielbassa *et al.* 2006), as well as comparing dental erosion/abrasion between human and bovine teeth (Amaechi 1999, Attin 2007, Meuran and Frank 1991, White 2010). According to Yassen *et al.* (2010), the results of the studies addressing enamel caries, there was inconsistent data relating to the use of bovine teeth as a substitute for human teeth. Furthermore, inconsistent outcomes were suggested regarding the use of bovine substrates as an alternative to human substrate in dental erosion studies (Yassen *et al.* 2011).

The present study will use bovine teeth, due to ease of practicality, ease of obtaining sufficient quantity and adequate quality. Additionally, as this study will use bovine and not human teeth, ethical approval will not be required, thus aiding the expediency of the study.

1.3 Plaque

In this *in vitro* study, dental plaque or oral bacterial microflora was not used. However, to fully understand and appreciate the process of enamel demineralisation and the caries disease process, it is pertinent to understand the role of plaque in such processes. Dental plaque is a complex biofilm consisting of a microbial community (Marsh 2006). Plaque can build up on any site within the oral cavity and is a natural physiological process. The microbial community of organisms within plaque are organised in a three-dimensional structure enclosed in a matrix of extracellular material derived from the cells themselves and their environment (Pretty *et al.* 2005).

Dental plaque formation occurs in progressive stages. These stages have been described as below (Marsh 2004):

- Formation of a pellicle: an acellular proteinaceous film, derived from saliva, which forms on a 'naked' tooth surface.
- Within 0-4 hours, singular bacterial cells colonise this pellicle. The majority of these bacteria are *streptococci* (*Streptococcus sanguinis, Streptococcus oralis, Streptococcus mitis*). In addition to the streptococci species, there are also *Actinomyces* species and Gram-negative bacteria.
- Over the subsequent 4-24 hours the attached bacteria multiple and grow resulting in the formation of distinct microcolonies.
- In 1-14 days, the *streptococcus*-dominated plaque changes to a plaque dominated by *Actinomyces*. This shift in the microbial population is known as microbial succession.
- In 2 weeks, the plaque has matured but there can still be site-to-site variations in the composition of the dental plaque. Each site is unique, which may explain the difference in lesion progression over different sites within the oral cavity.

Bacterial plaque is critical for the manifestation of the caries disease process in the oral environment. Some sites are more favourable for plaque retention as they encourage plaque retention and stagnation. These sites include enamel pits and fissures on premolars and molars, as well as approximal enamel smooth surfaces.

1.4 Dental Caries

Dental caries is a disease process involving localised chemical dissolution of the dental hard tissues. Caries can manifest on any tooth surface within the oral cavity where dental plaque is allowed to mature over time. This is as a result of the metabolic events taking place within the oral cavity.

Caries is a multifactorial process and for the disease process to occur there must be presence of a microbial community, fermentable carbohydrates, dental hard tissue and time (Keyes and Jordan 1963). In normal conditions, the oral environment will be at or above the critical pH of 5.5. The bacterial microflora within dental plaque is

metabolically active and these bacteria can be capable of fermenting a suitable dietary carbohydrate substrate (such as sucrose and glucose) to produce organic acid. This results in the pH of the dental plaque to fall below 5.5. This shift of the physiological state within the oral cavity can lead to demineralisation and repeated episodes of demineralisation results in the net loss of mineral leading to the manifestation of a carious lesion.

Caries is a complex disease process which can be affected by a number of factors such as the presence of a microbial community in the oral environment, fermentable carbohydrates, saliva composition, fluoride ions and time. The disease process is manifested as a dynamic process within the oral cavity and the caries process is a continuum which results from the repeated cyclic periods of demineralisation and remineralisation. There are other additional contributory factors which include social class, attitudes, education and behaviour (Keyes and Jordan 1963).

Cariogenic bacteria, mainly *streptococcus mutans* and *lactobacilli*, play a number of roles in the caries disease process (Kidd 2005). The bacteria can:

i) transport sugars and convert them to acid (acidogenic)
ii) Produce extracellular and intracellular polysaccharides which contribute to the plaque matrix; intracellular polysaccharides can be used for energy production and converted to acid when sugars are not available.
iii) Thrive at low pH (aciduric).

To allow for acid production, the presence of plaque and fermentable carbohydrates on the dental hard tissues is required for a period of time. It must be said that not all carbohydrates are equally cariogenic. Complex carbohydrates such as starch are relatively harmless because they are not completely digested in the mouth, but carbohydrates with low molecular weight (sugars) diffuse readily into plaque and are metabolised quickly by the bacteria (Marsh 2006). Due to this, many foods and drinks (especially energy and sports drinks) cause a fall in the plaque pH and thus cause demineralisation of dental enamel.

Saliva and fluoride play an important part in the physiological conditions in the mouth. Under normal physiological conditions, the teeth are bathed in saliva, which is supersaturated with calcium and phosphate ions (Marsh 2006). These ions are capable of remineralising teeth and help to prevent further progression of lesion formation. The widespread use of fluoride within toothpastes has been shown to result in a decrease in the prevalence of dental caries in most industrialised countries (Brambilla 2001). Fluoride ions (e.g., within Toothpaste) are adsorbed onto the hydroxyapatite crystals to from fluorohydroxyapatite which is more resistant to the organic acid produced by the cariogenic bacteria.

1.5 Erosion

Erosion is a term used to describe the process of gradual destruction of the surface of something, usually by electrolytic or chemical processes. In terms of the clinical term dental erosion, this describes the destruction of the dental hard tissues due to a chronic chemical process of dissolution caused by extrinsic acids, intrinsic acids or of an idiopathic nature. This process is usually painless and does not have any bacterial involvement (Imfeld 1996).

Extrinsic erosion is caused by exogenous acids, such as those within food and drink. Although there are natural acids within fruits and vegetables, usually in moderation, natural acids from natural dietary sources will not cause destructive damage to the dental hard tissues. Artificial sources of dietary acids will be those contained within beverages such as carbonated drinks or energy drinks. Energy drinks, and sports drinks to an extent, contain several erosive acids, such as phosphoric acid and ascorbic acid. It can be proposed that the consumption of these beverages places the consumer at an increased risk of dental erosion caused by extrinsic acids due to the acidic content within energy drinks.

Clinically, dental erosion is primarily a surface phenomenon, whereas dental caries originates as a subsurface demineralisation of enamel tissue (Pindborg 1970). Dental enamel solubility is pH dependent, and the rate at which apatite precipitates depends on certain factors such as calcium binding in saliva (Moss 1998). Under normal oral conditions, saliva contains calcium and phosphate ions to exist in a supersaturated

state at neutral pH. As the pH of the saliva decreases, it goes below the critical pH. For dental enamel, the critical pH is approximately 5.5 and pH lower than this value results in the enamel demineralisation. Conversely, an increase in the pH higher than 5.5 will result in the dental enamel remineralisation. As the critical pH of enamel is 5.5, any solution with a lower pH than 5.5 has the potential to cause acid erosion, particularly if the attack is lengthy and intermittent over time (Lussi et al 2004).

The extrinsic aetiology of diet has been extensively studied, however, the actual evidence linking a particular acidic food or beverage as the primary causative agent is limited (Moss 1998). However, the existing evidence strongly supports the detrimental role of acidic foods and beverages in dental erosion (Rytömaa *et al.* 1988). Apart from dietary causes, extrinsic erosion can also be caused due to environmental or occupational factors such as exposure to disinfectant agents within swimming pools, medication or lifestyle choices such as tooth bleaching (Lussi *et al.* 2004).

Tooth erosion due to intrinsic factors is due to the result of the dentition coming into contact with endogenous acids. This could be due to a health condition resulting in gastroesophageal reflux, chronic vomiting (due to bulimic eating disorder) or regurgitation. This would result in the oral cavity being exposed to gastric acid, and thus create a low pH, lower than the critical pH of 5.5. Repeated episodes of gastric acid reaching the oral cavity frequently and consecutively can lead to dental erosion (Moss 1998). Although uncommon, dental erosion can also have an idiopathic aetiology. In order to prevent dental erosion, it is important to know the aetiology, so steps can be taken to prevent erosion from materialising in the first place or preventing further destructive loss of enamel hard tissue (Moss 1998). It would be pertinent to investigate the effects of various energy and sports drinks in terms of their erosive potential on teeth.

In the initial stages of dental erosion, the process is manifested as gradual demineralisation of the enamel surface which in turn leads to softening of the enamel surface and consequent wear over time (Schlueter *et al.* 2011). As this erosive process progresses, there is a reaction from the dentine resulting in the formation of reactionary and reparative dentine. If tissue lost exceeds the reparative process, there will be

demineralisation of the tooth. Furthermore, due to the biological response to compensate for the loss of tissue, there is destruction of the dentinal tubules (Mjor 2001, Ganss 2006). There is an increasing awareness amongst consumers of acidic drinks about the erosive potential of these beverages. As a result, product manufacturers are under pressure to provide research concerning the erosive potential of their products (Pretty *et al.* 2003).

1.6 Energy and Sports Drinks

Energy drinks are soft drinks that contain higher levels of caffeine compared to other soft drinks, as well as containing a significant number of free sugars (although there are now low-calorie or zero-calorie versions available) whereas Sports drinks are aimed at improving physical performance by maintaining good levels of hydration during activities. These drinks are aimed to offer a variety of physiological benefits to the consumer, such as providing energy, supporting mental alertness and aiding physical performance. Typically, these drinks contain a high proportion of free sugars and have a low pH (Schneider *et al.* 2011). Despite the potential benefits on offer, the ingredients of these drinks can have negative implications for oral and general health and the consumption of energy and sports drinks has been shown to increase the risk of dental caries (Moynihan and Kelly 2014), dental erosion (Pinto *et al.* 2013), and obesity (Te Morenga *et al.* 2012). Furthermore, there is a known association between eating foods which are high in 'free' sugars and dental caries (Moynihan and Kelly 2014).

Despite the reported adverse effects of these drinks, they remain popular amongst children and adolescents (Kumar and Park 2015). The European Food Safety Authority (EFSA) commissioned a survey study in 2013 to gather data on the consumption of energy drinks. The survey involved 16 different member states, including the United Kingdom, and the survey study concluded that energy drink consumption amongst young people in the U.K. is higher than in any other European country (EFSA External Scientific Report 2013). Manufacturers are required by European Union law to label all energy drinks containing >150mg of caffeine per litre as "not recommended for children" (EFSA 2015). Despite the warning labels, children are still consuming these drinks; recent evidence shows that more than two thirds of UK children aged 10-17, and nearly a quarter of those aged 6-9, are energy drink consumers. (Department of

Health and Social Care: Consultation on proposal to end the sale of energy drinks to children 2016). adolescents (aged 10-17) who drink energy drinks are drinking, on average, 50% more than the EU average for that age group (Zucconi *et al.* 2013). Though some of these children may only have an energy drink occasionally, data tells us that a quarter of children who consume energy drinks will have three or more in one sitting (EFSA 2015).

Energy drinks typically contain active ingredients such as glucose, caffeine, taurine, or B-vitamins, and may also include other ingredients which are thought to be beneficial to health such as ginseng and other vitamins and minerals. The primary nutrient in most energy drinks is carbohydrate and/or caffeine. The most common form of added sugar is glucose, sucrose or high fructose corn syrup (Raizel *et al.* 2019). In addition to the high caffeine content of energy drinks, these beverages also contain high levels of sugar, comparable to that found in other soft drinks (Alsunni 2015). Given the high sugar content of energy drinks, as well as the low pH, such beverages have potential adverse health effects, such as increased risk of Type 2 Diabetes and obesity (SACN Report 2015, Nowak *et al.* 2018)

Published research has reported on wider general health and safety implications for consumers of energy drinks. Adult consumers of energy drinks frequently combine these drinks with alcoholic beverages (Oteri *et al.* 2003), which has can be a potentially fatal combination. Numerous case reports have linked the death of patients to the excessive consumption of energy drinks, with or without alcohol (Lehtihet *et al.* 2006, Berger and Alford. 2009). This risky combination has also been reported to have severe consequences for adolescents and young adults. A recent randomised control trial conducted in Germany concluded that energy drink consumption causes significant adverse changes in blood pressure, heart rate and glucose metabolism in young and healthy individuals (Basrai *et al.* 2019).

Components of energy and sports drinks

The potential functional benefits offered by energy and sports drinks are linked to their ingredients. There are a number of components of these beverages and their respective roles are discussed in the relevant sections below.

Water

Water is an essential part of a daily diet and adequate hydration is necessary for maintaining normal cardiovascular, thermoregulatory and other physiologic functions during physical exercise and routine daily activities (Schenider and Benjamin 2011). There are several factors which affect body euvolemia, such as diet, medications ill-health and chronic health conditions (Schenider and Benjamin 2011). Dehydration is caused by an imbalance body water intake and body water loss. There are a number of negative consequences of dehydration such as fatigue, impaired physical performance, cognitive changes and increased risk of heat illness (Petrie *et al.* 2004). Water is generally the most appropriate first choice for hydration before during and after physical exercise (Schenider and Benjamin 2011).

Carbohydrates

Carbohydrates are an essential and important source of energy for active children and adolescents (Schneider *et al.* 2011). Moderate consumption of carbohydrates along with balanced intake proteins, fat and other nutrients is part of a normal healthy diet. Energy and sports drinks often contain a very high amount of carbohydrates ranging from 21g to 34g per oz and this in mainly in the form of sucrose, glucose or high fructose corn syrup (Alsunni 2015). Regular consumption of these drinks containing such high levels of carbohydrates can substantially increase the risk for obesity in children and adolescents (Rodriguez *et al.* 2009).

In addition, fermentable carbohydrates (such as glucose and sucrose) play an essential role in the caries disease process. Caries is a multifactorial process and for the disease process to occur there must be presence of a microbial community, fermentable carbohydrates, dental hard tissue and time (Keyes and Jordan 1963). Consumption of carbohydrate containing energy drinks has been shown to be strongly associated with dental erosion (Marshall *et al.* 2003, Hasselkvist *et al.* 2009)

Caffeine

Caffeine is a central nervous system stimulant of the methylxanthine chemical group (Nehlig *et al.* 1992). Caffeine seems to interfere with adenosine in the brain at multiple sites, including the reticular formation. Adenosine is a naturally occurring xanthine in the brain that is used as a neurotransmitter at some synapses (Oteri *et al.* 2007). It has been suggested that caffeine may have a direct inhibitory effect on the sleep system (Porkka-Hieskanen *et al.* 1997). In addition to this, studies have also reported other effects of caffeine on the CNS such as an increase in alertness, reduction of fine motor coordination, headaches, dizziness and insomnia (Battig and Welzl 1993).

Guarana and Taurine

Guarana is a plant extract that contains caffeine (Australia New Zealand Food Authority 2001) and is marketed to increase energy, enhance physical performance and promote weight loss (Schneider *et al.* 2011). A single gram of guarana is equal to approximately 40mg of caffeine (Finnegan 2004). Energy drinks that contain guarana are a cause of concern due to the high caffeine content (Santa Maria *et al.* 1998). Taurine is a sulphur-containing amino acid present in high concentrations in mammalian plasma and cells (Schuller-Levis and Park 2003). It is essential for cardiovascular function, and the development and function of skeletal muscle, the retina and the central nervous system (Zucconi *et al.* 2013). Taurine is involved in a wide array of physiological phenomena (Oteri *et al.* 2007). It has been reported that taurine has an inhibitory effect on neurotransmission (Birdsall 1998).

Electrolytes

Electrolytes such as sodium and potassium are often found in energy and sports drinks (Schneider *et al.* 2011). Functional electrolyte requirements are sufficiently met by a normal balanced diet; therefore, energy and sports drinks offer little advantage over plain water for electrolyte maintenance (Ganio *et al.* 2007).

Vitamins and minerals

Energy and sports drinks often advertise a number of vitamins and minerals. However, these vitamins and mineral requirements are often met by maintaining a well-balanced

diet, therefore there is no advantage to consuming vitamins and minerals in energy and sports drinks (Schneider *et al.* 2011).

1.7 Obesity

Obesity is defined as abnormal and excessive fat accumulation that may impair health (World Health Organisation 2016). The most commonly used method of evaluating healthy weight in adult is by use of the Body Mass Index (BMI). BMI is defined as an individual's weight in kilograms divided by the square of their height in metres (kg/m^2). The World Health Organisation defines obesity as a BMI greater than or equal to 30 (World Health Organisation 2016).

Aetiology of obesity

The common causative factors are the consumption of highly calorific foods and drinks in combination with a lack of physical exercise. This combination leads to an imbalance in the number of calories consumed and calories that are burned off through physical exercise, leaving the excess energy which is then stored by the body as fat.
(Jebb 1997).

Prevalence of obesity

In recent years, there has been an increase in the global prevalence of obesity and associated health risks. Since 1975, worldwide obesity has nearly tripled and statistics as of 2016 show that there are more than 1.9 billion adults who are overweight, of which 650 million are considered obese (World Health Organisation 2016). Previous studies have shown that consumption of sugar-sweetened beverages as compared with non-calorically sweetened beverages, resulted in greater weight gain and increased BMI (James *et al.* 2004, de Ruyter *et al.* 2012, Ebbeling *et al.* 2012).

In July 2015, the Scientific Advisory Committee on Nutrition (SACN) published a report on Carbohydrates and Health, which provided recommendations to the U.K. government on the population intakes of total carbohydrate, sugars and fibre in the U.K. (SACN 2015). This report confirmed the association with sugar-sweetened beverages (such as energy drinks) and weight gain, risk of type 2 diabetes and dental

caries. Based on prospective cohort studies in children, SACN concluded that higher consumption of sugars, and sugar- containing foods and beverages, is associated with a greater risk of dental caries.

1.8 Mineral content evaluation techniques

1.8.1 Microradiography

Microradiography is a destructive technique which quantifies the mineral content of dental hard tissue by measuring the attenuation of X-rays transmitted through a section by comparison with a reference aluminium step wedge (Schlueter *et al.* 2011). There are two techniques for microradiography: Transverse Microradiography (TMR) and Longitudinal Microradiography (LMR).

1.8.2 Transverse Microradiography

The TMR technique was first described by Edgar de Josselin de Jong (de Josselin de Jong *et al.* 1987) and it has since become a widely-used technique in dental cariology research to quantify dental hard tissue mineral content. Arends and Ten Bosch (1992) describe the accuracy of TMR for mineral loss ΔZ is 200 vol%μm and lesion depth (L_d) is approximately 5 μm. However, it has been reported that incorrect measurements can be created if the lesion is not uniform (Garry *et al.* 2017).

The foundation for TMR analysis is based on the measurement of x-ray absorption of the sectioned tooth sample compared with an exposed standard. The differing levels of x-ray absorption by the sectioned samples and the step-wedge standard affects the optical density of the developed film. The aluminium step-wedge has ascending layers of aluminium, ranging from 0 to 11 layers, with each aluminium foil being approximately 0.275mm in thickness. The mineral content of the sectioned samples can be established by using a bespoke software and image analysis tool using a digital single lens resolution camera (CCD). This allows for generation of mineral content profiles for the samples (Garry *et al.* 2017). This technique allows for Mineral Loss ΔZ (vol%μm), Lesion depth L_d (μm) and Lesion width L_w (μm) to be obtained from the analysis of the mineral content profiles. Mineral loss is the difference between the mineral content of the baseline sound enamel and the sectioned sample. Lesion depth and Lesion width are obtained from the mineral distribution from the samples (Garry *et al.* 2017).

The TMR technique for mineral content evaluation has many advantages. It is a valid and reliable technique for detecting demineralisation and is considered to be the gold standard for quantitative measurement of mineral content levels (de Josselin de Jong *et al*. 1995). A major disadvantage of the TMR process is that it requires destruction of the sample and therefore limiting its use (Ten Bosch and Angmar-Mansson 1991). Once the samples have been sectioned for analysis using TMR, they are no longer viable for any other analytic tools or purposes. Additionally, TMR is a technique sensitive process and presents technical difficulties. These may include the handling and sectioning of small enamel samples (samples should be between 80-100μm) and in creation of the required parallel sections. This requires meticulous and precise sample preparation and careful handling during all stages of TMR analysis. Other limitations include an inability to measure less than 10 μm from the anatomical surface due to the inherent curvature of the samples (White *et al.* 1992). Despite these limitations TMR is generally accepted as the gold standard in direct quantification of mineral change. A comparison of TMR results with QLF is routinely used for validation (Garry *et al.* 2017).

1.8.3 Quantitative Light-Induced Fluorescence Digital (QLF)

The use of Laser Auto-Fluorescence for the detection of early dental caries was first described by Bjelkhagen and Sundström (Bjelkhagen and Sundström 1981). This technique has been further developed into a quantitative method for the evaluation of mineral loss (Hafström-Bjorkman *et al.* 1992). QLF is a non-destructive optical technique which uses the natural fluorescence of teeth to assess early carious lesions in enamel. When a tooth becomes carious the fluorescence radiance at the location of the caries lesion decreases. The fluorescence image of enamel with incipient lesions can be digitised and then the fluorescence loss in the lesion can be quantified in comparison to the fluorescence radiance level of sound enamel (van der Veen and de Josselin de Jong 2000). QLF has many advantages. Firstly, it is a non-destructive method for the quantification of enamel mineral loss. Secondly, this technique allows for the longitudinal assessment of lesions. This is particularly advantageous in studies assessing remineralisation of early carious lesions. QLF can also be used in vivo and *in vitro*.

QLF has been shown to be sensitive and reproducible. A study conducted by Elton *et al.* (2009) suggested that QLF is highly effective for measuring the subsurface loss of mineralisation but is less effective at measuring lesion depth. QLF ability to measure demineralisation has been shown to be comparable to the more destructive Transverse Microradiography technique (Lovel *et al.* 2008).

1.9 Aims and objectives

1.9.1 Aims

The primary aims of this study were:

1. To develop a novel *in vitro* pH cycling model which is representative of the daily dietary cycle with respect to the pH fluctuations and challenges encountered in the oral environment.
2. To confirm that the novel cycling model does not result in enamel demineralisation by conducting *in vitro* experiments and subsequent analysis of mineralisation of sound bovine enamel exposed to a negative control drink.
3. To investigate the effects of energy and sports drinks on sound and demineralised bovine enamel utilising the novel cycling model
4. To assess and analyse the differences between the energy and sports drinks in terms of their effects on enamel mineralisation on both sound and demineralised samples.

The outcome measure was enamel mineral loss quantified as ΔF (% loss of fluorescence) and ΔZ (vol%μm) measured by Quantitative Light-Induced Fluorescence Digital (QLF) and Transverse Microradiography (TMR) respectively.

1.9.2 Objectives

The objectives of the current study were:

1. To conduct *in vitro* cycling experiments to design a cycling protocol for a novel model which can be regarded as representative of the environment within the oral cavity over a 24-hour period with respect to pH fluctuations and challenges.
2. To conduct chemical analysis of Red Bull energy drink, Monster energy drink and Lucozade sports drinks to ascertain their potential to cause enamel dissolution and demineralisation.
3. To quantify the study data to assess changes in mineralisation of sound and demineralised enamel samples before and after exposure to Red Bull, Monster and Lucozade Sport.
4. To quantify changes in mineralisation of sound and demineralised enamel samples before and after exposure to Red Bull, Monster and Lucozade Sport compared to positive and negative controls.

5. To understand the effects of Red Bull, Monster and Lucozade consumption on sound and demineralised enamel after 14 days of *in vitro* cycling.

1.9.3 Null hypothesis

There is no significant difference in enamel mineral loss in sound bovine teeth exposed to either a test drink or control drink after 14 days of *in vitro* cycling.

There is no significant difference in enamel mineral loss in demineralised bovine teeth exposed to either a test drink or control drink after 14 days of *in vitro* cycling.

1.9..4 Research Questions

1. Does exposure to Red Bull, Monster and Lucozade Sport result in enamel mineral loss in sound bovine teeth *in vitro*?
2. Does exposure to Red Bull, Monster and Lucozade Sport result in enamel mineral loss in demineralised bovine teeth *in vitro*?
3. Is there a difference between the Red Bull and Monster energy drinks when compared to Lucozade sports drinks in terms of enamel mineral loss after 14 days of *in vitro* cycling?

1.9.5 Rationale

The consumption of sugar-sweetened beverages such as energy drinks is now becoming a growing public health concern. In recent years, there has been an increased awareness of the damaging effect that energy drinks can have on not only oral health, but general health as well. A recent cross-sectional study investigating oral health-related behaviours reported by elite and professional athletes (Gallagher *et al.* 2019) showed the detrimental effect energy drinks can have on the dentition, despite maintaining good oral health practices and behaviours.

Taxing sugar-containing beverages is now implemented in many countries as a strategy to reduce the purchase and consumption of sugar to tackle obesity. In an effort to tackle the increasing prevalence of childhood obesity within the United Kingdom (U.K.), the U.K. government introduced the Soft Drinks Industry Levy in April 2018. This legislation, often referred to commonly as the ‘Sugar Tax’, puts a charge of

24 pence on drinks containing more than 8g of sugar per 100ml and a charge of 18 pence for drinks containing 5-8g of sugar. This levy was introduced as part of the UK

Governments' childhood obesity strategy, by aiming to reduce sugar consumption by persuading manufacturers to reformulate their beverages and avoid paying the levy. Public Health England, which is an executive agency of the Department of Health and Social care (U.K. Government) called for a 20% cut in sugar content within food produce by 2020 (Public Health England publications gateway number 2016677PDF 2017). At a time where there is an increased awareness of dietary factors affecting general and oral health, it is important to fully understand the potential damaging effects of energy drinks. This study investigated the effects of the two most popular energy drinks and the best-selling sports drink in the U.K. and aimed to quantify the effects on the consumption of these drinks on sound and demineralised bovine enamel.

It is hoped that the data and conclusions from this study can be used for future investigations and research assessing the impact energy drinks have on the human dentition. Dental caries is a largely preventable disease; however, it remains very common amongst the U.K population and there is now an increased awareness of the direct association between sugar-containing beverages such as energy drinks and dental caries. The U.K. population has a very high consumption of energy drinks, and the current evidence suggests that the consumption of sugar-sweetened beverages (including energy drinks) is highest amongst adolescents aged 13 to 18 years old (Ng *et al.* 2012). If this trend continues, there is a risk of potential health consequences, which ultimately will increase the burden on an already stretched and over-burdened National Health Service.

Chapter 2 Materials and Methods

2.0 Materials and Methods

2.1 Ethical Approval

This study did not use human tissue, therefore ethical approval was not required. This research was an *in vitro* study involving bovine teeth.

2.2 Design

In vitro experimental case control study.

2.3 Sample source

Bovine teeth were obtained from a local abattoir which maintains a longstanding working relationship with the School of Dentistry, University of Liverpool. Following slaughter, the mandibles of cattle were sectioned and separated from the facial skeleton to facilitate the extraction of bovine teeth. Permanent bovine mandibular incisor teeth were removed and acquired for use in this study.

After acquiring sufficient quantity of bovine teeth, they were transported to the research laboratory for safe storage. The teeth were stored in distilled water at 3°C inside a laboratory fridge.

2.4 Setting

Department of Health Services Research at the Royal Liverpool University Dental Hospital (RLUDH).

2.5 Methods

2.5.1.1 Sample selection

After acquiring sufficient quantity of bovine mandibular incisors from a local abattoir, a visual inspection was performed in the first instance to assess the suitability for use in this study. The inclusion criteria for the bovine teeth included permanent mandibular incisor teeth that were free from pathology (without any tooth wear, hard tissue fracture or caries). Primary bovine teeth, bovine molars and any teeth affected by bovine were excluded from this study.

Following the initial visual examination, suitable bovine teeth were viewed under a Quantitative Light Fluorescence (QLF) camera to assess for any evidence of hard tissue pathology which were not evident by visual examination. Any bovine teeth which

exhibited signs of caries, crown fracture or any hard tissue pathology with the use of the QLF camera were discarded and not used in this study. The QLF camera offered a more detailed inspection in comparison to the visual examination. Only bovine teeth which satisfied the inclusion criteria after visual and QLF inspection were selected for use in this study. By ensuring only sound bovine teeth were selected for this study, this has increased the reliability and validity of the results, as a baseline standard was established in terms of quality of teeth selected. To ensure that sufficient quantity of bovine teeth were acquired for the entirety of the experimental aspects of this study, a minimum of 150 bovine teeth were selected as being suitable for use. These teeth were then sectioned to obtain the enamel samples as described in the following sections.

2.5.1.2. Removal of soft tissue

Once pathology-free samples have been identified, any soft tissue attachments were removed from the teeth using a Number 15 scalpel (Swann-Morton, Sheffield, England, United Kingdom, as displayed in Figure 2.1)

Figure 2. 1 Surgical steel blade of removing soft tissue attachments

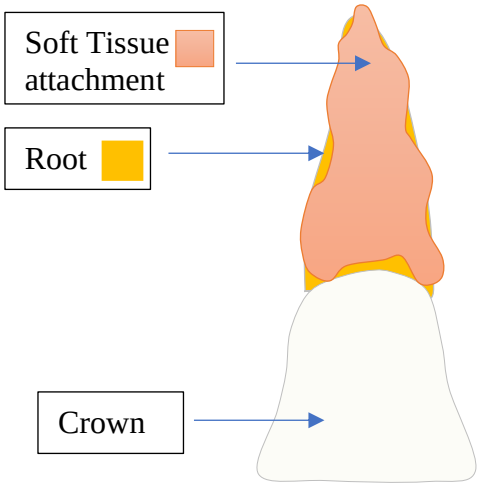


Figure 2. 2 Diagrammatic illustration of bovine tooth prior to removal of soft tissue attachment

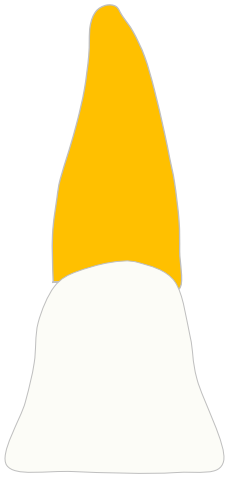


Figure 2. 3 Diagrammatic illustration of bovine tooth after removal of soft tissue attachment

2.5.1.3. Creation of flat enamel surface

The first step for the preparation of the enamel surface involved the creation of a flat enamel surface on the labial aspect of the bovine teeth. This was undertaken with use of the EcoMet 30 grinding and polishing machine (Buehler, Illinois Tool Works, Lake Bluff, Illinois, United States of America, as displayed in Figure 2.4).

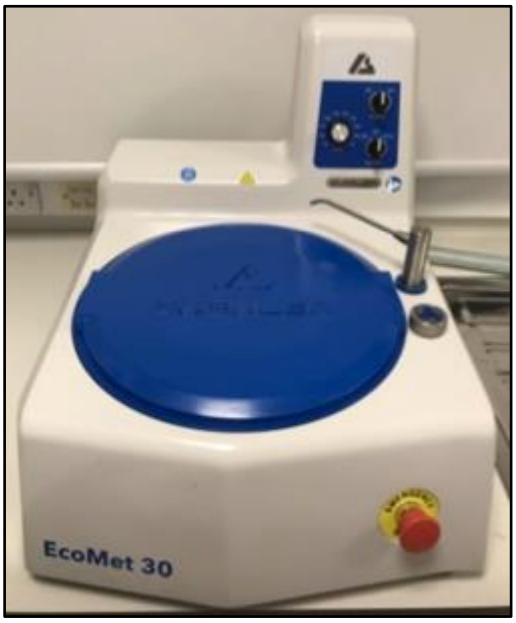


Figure 2. 4 EcoMet 30 Manual Polishing machine

The EcoMet 30 is a manually operated grinding and polishing machine. It allows the operator to polish the samples under a cooling water source. The manually operated EcoMet 30 provided ease of use and allowed for a gradual polishing process. The creation of a flat enamel surface involved a 3-step process using the EcoMet 30 grinding and polishing machine. Three polishing discs at differing levels of coarseness were used to initially create a flat surface and then a highly polished surface for experimental use. The initial flattening of the labial surface of the crown involved using a very coarse P220 level grit and this was used to flatten the labial surface of the bovine tooth. Once a flat labial surface was produced, the polishing coarseness was changed to a lower level of coarseness, and this involved use of the P1200 level grit. The final step involved use of the P4000 level grit to give a final highly polished finish to the labial enamel surface.

For this study, it was essential to create a flat or near-flat enamel surface for experimental purposes as well as having a minimum depth of enamel on all the

samples (minimum of 1.0mm enamel). This study involved the use of Transverse Micro-Radiography (TMR) analysis, it was important to have a flat enamel surface to allow accurate use of the TMR technique.

2.5.1.4. Sectioning of root fragment

After the bovine teeth labial surfaces were ground and highly-polished, the root fragment was sectioned from the coronal fragment. The root of the tooth was not of any use in this study therefore it was sectioned at this point of the sample preparation. The sectioning was undertaken using an Isomet Low speed cutting saw (Figure 2.5). After sectioning of the root, this left the crown of the tooth intact (Figure 2.7) which would be then further sectioned to produce the enamel blocks for experimental purposes.

Figure 2.5 Isomet Low speed cutting saw

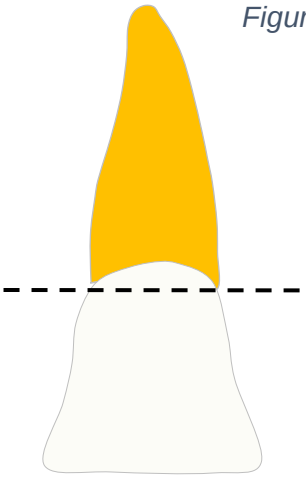


Figure 2.6 Diagrammatic illustration to demonstrate sectioning of root of bovine tooth

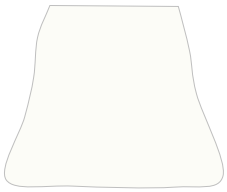


Figure 2.7 Diagrammatic illustration to demonstrate the crown of bovine tooth after root sectioning

2.5.1.5. Enamel sample creation

The next step involved further sectioning the highly polished labial surfaces of the bovine crowns into tooth samples which were used in the experimental stages of this study. The sample dimensions were 5mm (width) x 4mm (length) (Figures 2.8-2.10).

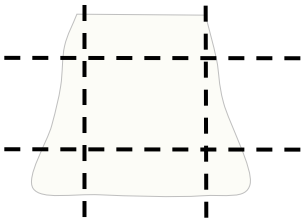


Figure 2.8 Diagrammatic illustration of crown sectioning

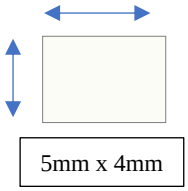


Figure 2.9 Diagrammatic illustration of sectioned sample

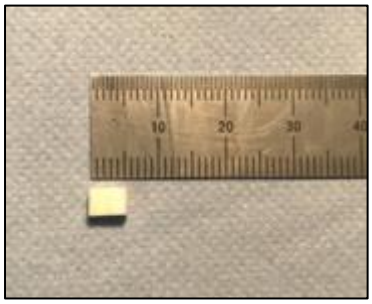


Figure 2.10 Sectioned sample

The samples were precisely cut into the blocks using the WELL Precision diamond saw (WELL Diamond Wire Saws, Crêt-Vaillant, Le Locle, Switzerland, as shown in Figure 2.11). The diamond wire saw produces a clean cut and leaves the sectioned bovine enamel blocks with precise cutting surfaces which are free from rough edges. The WELL diamond wire saw cuts the samples with cool water to preserve the tooth surface and prevent overheating and damage to the tooth sample. The wire diameter is approximately 0.17mm, which must be taken into account when sectioning teeth, both for the bovine enamel sample creation as well as for sectioning the samples for TMR analysis.

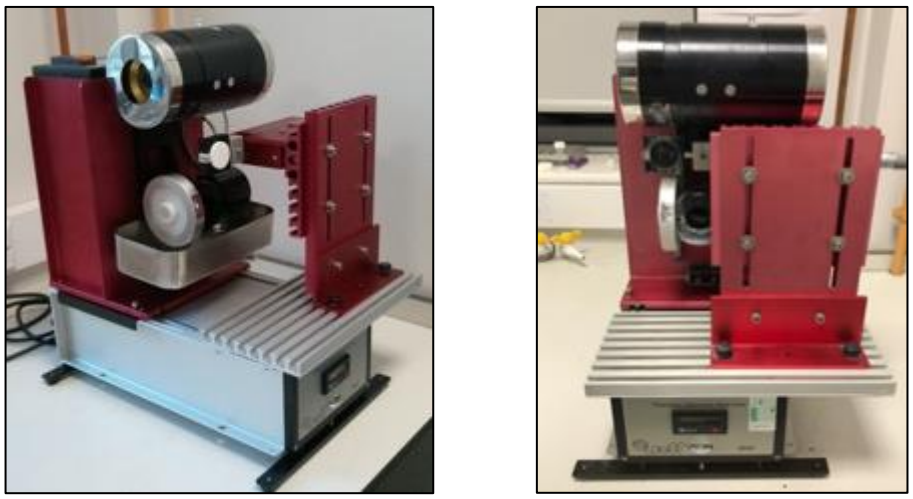


Figure 2.11 WELL Precision Diamond Wire Saw

2.5.1.6 Pilot study enamel samples

The samples used in the pilot study to develop a novel cycling model were 5mm x 4mm in dimension. Once these samples were created, each individual sample were initially cleaned with ethanol to remove any surface contaminants and left to air dry. In order to protect the baseline enamel, the outer margins and all edges of each sample was painted with an acid-resistant nail polish (MaxFactor Glossifinity 110 Clear), leaving a central exposed window (approximately 2mm x 2mm) for experimental use (Figure 2.12) . By protecting the margins and edges of the sample with an acid-resistant nail polish the baseline enamel, this allowed comparison of the baseline enamel surface with enamel surface exposed to acidic challenges.

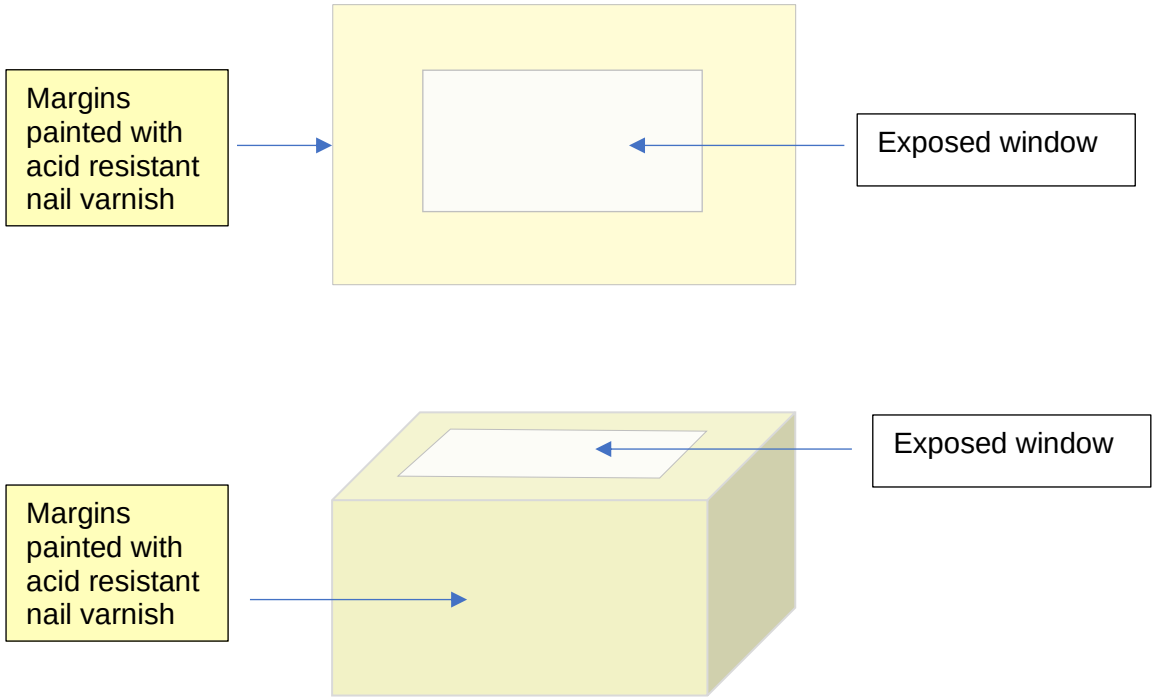


Figure 2.12 Diagrammatic illustrations of enamel samples with outer margins and edges painted with acid-resistant nail polish

2.5.1.7 Main study enamel samples

Prior to the main study, each 5mm x 4mm sample was further sectioned in half to produce a 2.5mm x 2mm pairs (Figure 2.13). This allowed one pair to be allocated to the sound baseline enamel groups and one pair to be allocated to the demineralized baseline groups. For the main study, 75 samples (5mm x 4mm) were selected and subsequently further sectioned 75 pairs of 2.5mm x 2mm samples (total number of samples = 150). The approach to create sample pairs was novel and this allowed for one pair to be kept sound and the other pair to be used to create an artificial carious

lesion before the experiments with the test drinks, thereby allowing for assessment and analysis of the test drinks on both sound enamel and enamel with early demineralisation. At the time of writing, such an approach to studying test drinks has not been described in the literature.

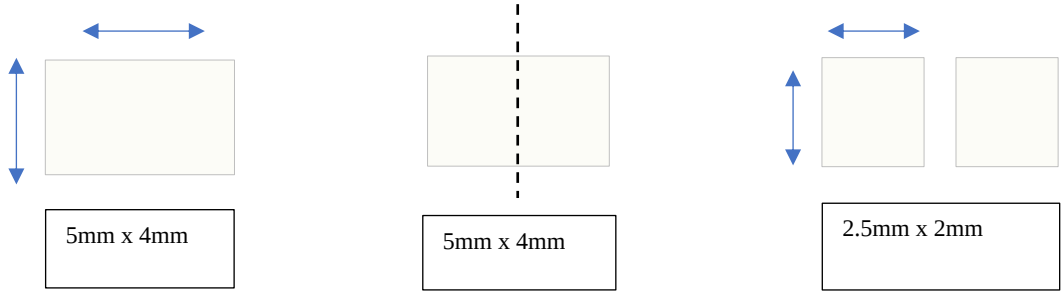


Figure 2.13 Diagrammatic illustration to demonstrate the sectioning of samples into pairs for the main study

Similar to the samples used in the Pilot studies, the paired samples for the main study had acid-resistant nail varnish painted on the margins and edges to protect the baseline enamel from the acidic challenges (Figure 2.14)

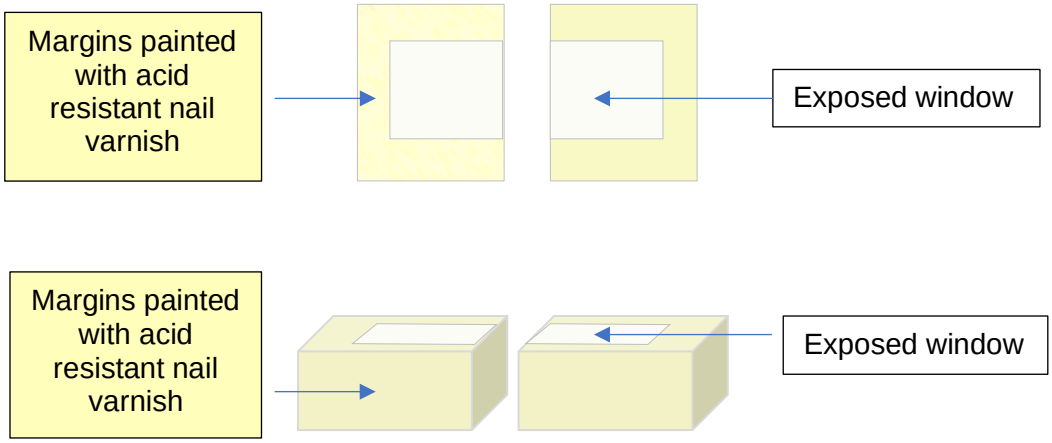


Figure 2.14: Diagrammatic illustration to demonstrate nail polish painted on margins and edges of paired samples

For the main study, each enamel sample was allocated a number to allow use of a number tracking system. This allowed for randomisation of the samples into the respective groups and also ensured that each pair was kept together for group allocation. One pair would remain sound at baseline and the other pair would have an artificial carious lesion produced prior to the start of the main study (Figure 2.15).

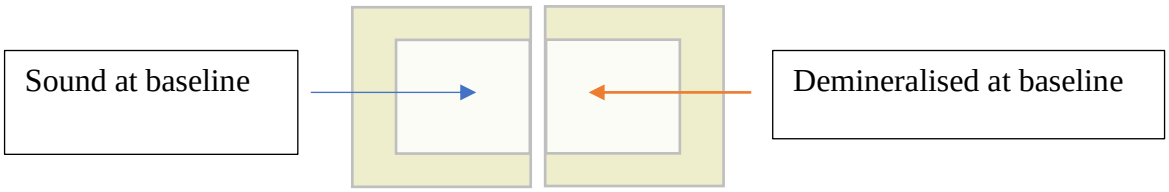
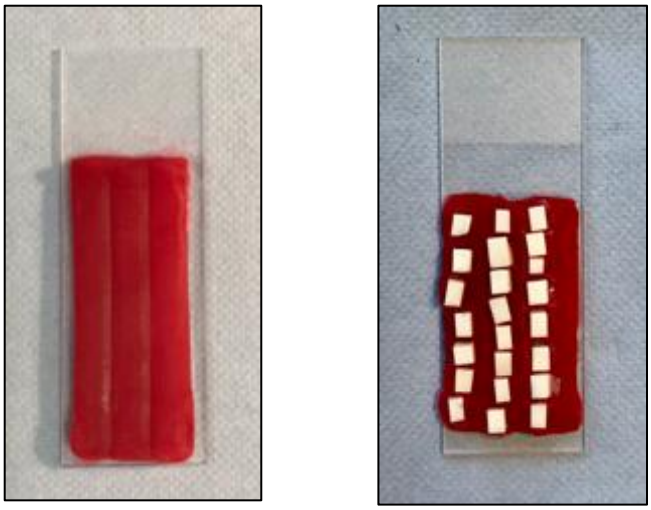


Figure 2.15: Diagrammatic illustration of paired enamel samples with one sound sample and one demineralised sample prior to the start of the main study

2.5.1.8. Creation of artificial carious lesions for main study

The 75 pairs of enamel samples were numbered and one pair from each enamel sample was used to create an artificial carious lesion on the exposed window. These samples underwent artificial demineralisation and were subsequently used in the main study to assess the effects of the test drinks and control drinks on demineralized enamel.

After sectioning and then painting with acid-resistant nail varnish, the demineralized samples were prepared for the artificial demineralisation process. Clear glass microscope slides (BDH, Dubai, United Arab Emirates) were used for placement of the enamel samples. The glass slides had one surface covered in red dental carding wax (Kemdent Works, Swindon, United Kingdom) to allow placement and fixation of the enamel sample onto glass slide. Each enamel sample was carefully placed within the carding wax and a number tracking system was used to ensure each sample was identifiable at all stages. The number tracking system not only ensured that the enamel samples were identifiable throughout the experimental stages of this study but also so that each pair (1 x sound sample, 1 x demineralised sample) were allocated correctly to their respective groups.



A B

Figure 2.16: A - Glass Microscope slides with carding wax to allow placement and fixation of enamel samples for artificial demineralisation. B - with samples placed on glass slide

Plastic lab containers (75ml) were filled with an artificial demineralisation solution at pH 4.5. The glass slides and samples within were accurately numbered and each glass slide (x 5 in total) was placed into a separate container. The demineralisation protocol involved immersing the enamel samples with the artificial demineralisation solution for 18 hours at 37 degrees Celsius (stored in an incubator which was set to 37 degrees Celsius).

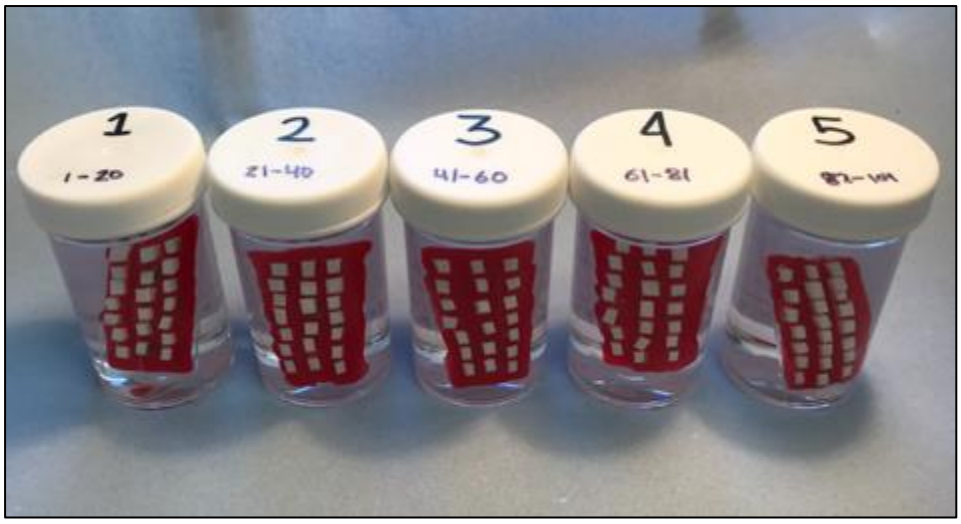


Figure 2.17: Enamel samples undergoing artificial demineralisation

After 18 hours, the enamel samples were removed, rinsed thoroughly with distilled water and left to air dry. Following this, they were analysed using QLF and TMR to for baseline demineralisation values.

At this stage, QLF and TMR techniques were utilised to detect presence of a demineralised lesion. The added advantage of using QLF at this stage is that any samples with lesions which are of poor quality or uneven distribution of demineralisation on the exposed window surface, can be identified and excluded from the main study. This will ensure that the testing is as accurate and reliable as it can be. If there is an absence of a subsurface lesion, the samples will be excluded from the main study.

After all baseline QLF and TMR values were recoded, each of the 75 pairs was then randomly allocated to one of 5 groups. The method of randomization was simple randomisation. To eliminate any selection bias, one-way ANOVA was performed to ensure that there were no significant differences for baseline mineral loss values amongst the 5 groups.

2.5.1.9 Placement of enamel samples for Pilot and Main study cycling

For both the pilot and main study, the technique for placing the samples for experimentation was the same. The samples were mounted on a centrifuge tube using carding wax (Kemdent) (Figure 2.18) on the outside to allow placement and fixation of the samples. The centrifuge tube was secured onto a bespoke placement rack for the cycling robot. The carding wax was preferred for mounting the samples on the centrifuge tube as it is non-reactive with the solutions as well as providing practical advantages such as ease of placement of the samples within the wax.

Figure 2.18: Tooth carding wax used for placing and fixing samples onto centrifuge tubes

2.5.2 Chemical Analysis

2.5.2.1 pH

Energy drinks, and sports drinks to some extent, are known for their acidity. The study test drinks were assessed for their pH values. pH is a logarithmic measure of the concentration of free hydrogen ions in a solution (Tyl and Sandler 2017). This involved using a pH electrode (pH meter 3305 Jenway). The electrode was calibrated at the beginning of each trial session using standard buffers of pH 4.0 and 7.0. The test drinks were stored at room temperature. One hundred (100) millilitres (ml) of each newly opened energy drink was placed in a beaker and left for 1 hour to allow the solution to de-gas. Thereafter, 100ml of each respective drink was stirred using a non-heating magnetic stirrer and the pH value was recorded when stable reading was evident on the pH meter. To ensure a reliable value, pH values for each drink was recorded in triplicate and the mean value was used to quantify the pH value for the respective drink. This method has been used in previous studies investigating the erosive potential of a range of diluting drinks (Cairns *et al.* 2002).

2.5.2.2 Titratable acidity

The test drinks were assessed for Titratable Acidity (TA). TA measures the total acid concentration in a solution (Tyl and Sandler 2017). This involved the neutralisation of the respective drinks with the addition of 1M Sodium Hydroxide (NaOH) until a pH 7.0 value was achieved. A newly opened container (aluminium can or plastic bottle) of

each test drink was poured into a large beaker and allowed to degas for one hour. Thereafter, 100ml of the test drink was poured into a beaker with a small magnetic stirrer and pH electrode placed within the beaker. The beaker was placed on a magnetic stirring machine. A burette was used and filled with 100ml of 1M NaOH. The burette allowed accurate and incremental addition of 1M NaOH to the drink. With small and incremental addition of 1M NaOH, the pH recording was carefully monitored using the pH electrode and pH meter until a pH value of 7.0 was achieved. The amount of 1M NaOH required to reach this value was subsequently recorded. The set up the TA apparatus is demonstrated in Figure 2.19..

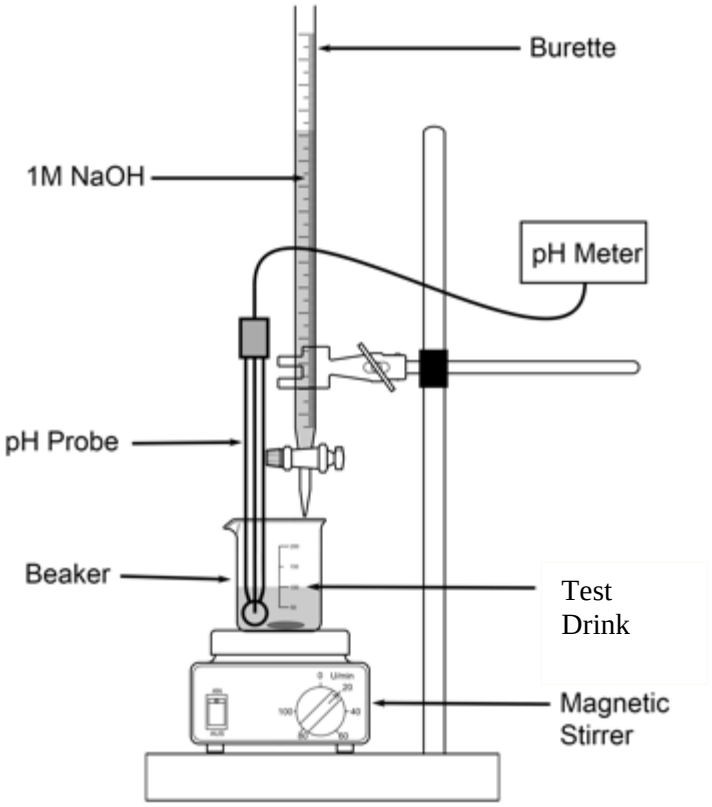


Figure 2.19: Titratable acidity apparatus

The TA values were recorded in triplicate and the mean value was used for each drink. This analysis was performed prior to the pilot and main studies. The volume of sodium hydroxide added will be plotted against pH and the amount required to reach pH value of 7.0 will give the measure of the titratable acidity of each of the energy drinks used in this study (Cairns *et al.* 2002)**.**

2.5.2.3 Fluoride analysis

Fluoride analysis was conducted for the test drinks. This was undertaken using an Ion Selective Electrode (Oakton by Cole-Parmer Combination Ion-Selective Electrode). The fluoride electrode consists of a sensing element bonded into an epoxy body. When the sensing element is in contact with a solution containing fluoride ions, an electrode potential develops across the sensing element. This potential, which depends on the level of free fluoride ion in solution, is measured against a constant reference potential using a digital pH/mV meter or Ion-Selective Electrode (concentration) meter.

The potential corresponding to the level of fluoride ion in solution is described by the Nernst equation E=Eo + S* log (A). Total Ionic Strength Adjustor Buffer (TISAB) is added to all fluoride standards and samples so that the background ionic strength is high, fluoride is decomplexed and the pH of the solution is correct.

Following pH, titratable acidity and Fluoride analysis have been undertaken, the samples are ready to undergo experiments. For the purposes of this study, the samples will not have any attachments (such as fixed orthodontic appliance) bonded onto the enamel surface. This will maximise the enamel surface area available for testing.

2.5.3 Test Drinks

In this study, the enamel samples were divided into one of 5 groups. Three (3) of these were test drink groups and two (2) were control drinks. The 3 test drinks included Red Bull energy drink, Monster energy drink and Lucozade sports drinks. These are 3 of the most popular and best-selling energy and sports drinks in the U.K. (Clapp *et al.* 2019, Statista 2021).

Test Drink	Classification	Manufacturer	Market share
Red Bull (Original)	Energy drink	Red Bull GmbH, Vorarlberg, Austria	38% (MNST 2019)
Monster (Original)	Energy drink	Monster Beverage, Corona, U.S.A	35% (MNST 2019)
Lucozade Sport (Orange)	Sports drink	Ribena Suntory Limited, U.K.	Unknown

Table 2. 1 Test drink classifications and manufacturers

A B C

Figure 2. 20 A – Red Bull energy drink, B – Monster energy drink, C Lucozade sports drink

	Red Bull	Monster	Lucozade
Energy	195kJ (46kcal)	201kJ (47kcal)	118kJ (28kcal)
Carbohydrates	11g	12g	6.5g
Carbohydrates of which sugars	11g	11g	3.6g
Fat	Negligible	Negligible	Negligible
Salt	0.1g	0.19g	0.13g
Protein	0g	Negligible	Negligible
Caffeine	32mg	32mg	0mg

Table 2. 2 Test drinks nutritional information per 100ml

Table 2.2 demonstrated the high carbohydrate content per 100ml of each test drink. In addition, both Red Bull and Monster energy drinks have high caffeine content. Both of these two drinks have a high caffeine content warning on their respective packaging with advisory comments that these drinks are not recommended for children or breastfeeding women and should be consumed moderately.

Overall, in the main study there were 5 groups in total, 3 test groups and 2 control groups. The groups are detailed in Table 2.3:

Role	Drink
Test drink	Red Bull Energy
Test drink	Monster Energy
Test drink	Lucozade Sport
Positive control	Coca-Cola
Negative control	Distilled deionised water

Table 2. 3 Test and Control drinks

2.5.4 Cycling robot

To perform the cycling methodology, this study used the Zinsser Analytic Cycling robot (Zinsser Analytic, Frankfort, Germany). This is an automated cycling robot (Figure 2.21) which is programmed using a software to perform precise cycling protocols. In this study, the robot was coded and programmed using a computer software to carry out the 24-hour cycling methodology.

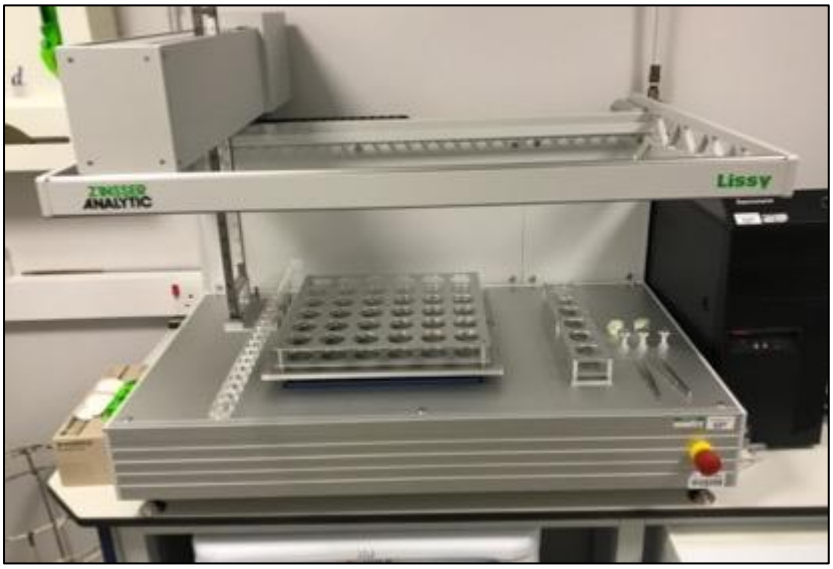


Figure 2.21 Zinsser Analytic Cycling Robot

The advantage of this cycling robot was the ease of practicality it offered. The cycling robot was programmed to run the cycling models over 24 hours, without the need for the operator to be present during the active cycling. Each cycle can be programmed to run to accurate pre-determined parameters such as total number of cycles, duration of cycle, agitation of the sample within the solution and washing with distilled de-ionised water (dH2O). The functionality and methodology related to the Zinsser cycling robot is discussed further in Chapter 3.

2.5.5 Blinding , Randomisation, and Group Allocation of samples

In the first pilot study, the experimental group samples were blinded from the primary investigator during the analysis stage. It is advantageous to blind the operator, data collector and data analysts to reduce the risk of bias. This will further aid in maximising the validity of this study. Blinding was however not possible for the main study samples due to practicality limitations. For the main study, the samples were numbered and then randomly assigned to one of 5 groups with equal numbers within each group (n= 15 paired samples/group). The numbered pairs of enamel samples were kept together throughout the main study and subsequent analysis.

2.6 Outcome measure

The outcome measure was enamel mineral loss quantified as ΔF (mean fluorescence loss) and ΔZ (vol%μm) measured by Quantitative Light-Induced Fluorescence Digital (QLF) and Transverse Microradiography (TMR) respectively.

2.7 Methods of Mineral Loss Assessment and Analysis

Dental caries and dental erosion can be diagnosed by assessing the change in mineral content within a sample. The protected baseline enamel serves as a sound baseline value and the enamel within the exposed window of the sample can be directly compared to the sound baseline to assess for changes in mineral content. Quantitative methods such as TMR and QLF analysis allows for changes between a protected and exposed surface within an individual sample to be detected and also allows for the assessment of carious lesion progression (Ten Bosch and Angmar-Månnson 1991). This study used two methods for quantitative analysis for mineral loss: QLF and TMR.

2.7.1 Quantitative Light-Induced Fluorescence Digital (QLF)

As QLF is a non-destructive technique, this was carried out prior to TMR analysis and all QLF images are captured in a dark room. QLF images of all samples in the pilot and main studies were captured using the QLF Biluminator system (Biluminator, Inspektor Pro Research Systems, Amsterdam, Netherlands) which was attached to a DSLR camera (Cannon 5500D, Canon, Tokyo, Japan). The Biluminator contains both blue LEDs (x6) and white LEDs (x4). When the image is being captured the blue LEDs emit a blue light (405nm) which is directed towards the enamel sample and excites the enamel and causes it to fluoresce. The white LEDs emit a white light (6500k) on the enamel sample. For each sample, both a white and blue light image was captured. The blue light image allowed for detection of a loss in fluorescence within the enamel sample. All settings for the images were standardised (blue light images: 2592 x 1728px, 1/40s shutter speed, 8.0 aperture, daylight white balance, ISO 1600, white light images: 2592 x 1728px, 1//40 shutter speed, 11.0 aperture, manual white balance, ISO 160). The vertical distance between the sample and camera lens was kept consistent for all samples.

Figure 2.22: Biluminator and DSLR set up for QLF imaging

Figure 2.23: Profile view of QLF image capture set up

After capturing images, the QLF analysis was carried out using a custom software (QLF Analysis Software QA2 v 1.26, Inspektor Pro Research Systems, Amsterdam, Netherlands). The software was used to draw an area on marginal sound enamel around the central exposed enamel window. This allowed a direct comparison between the preserved marginal enamel and central exposed enamel surfaces in

order to detect differences in fluorescence. A decrease in the fluorescence value between the exposed window and sound enamel was recorded as a loss of fluorescence (ΔF) (measured in %). After all images were analysed using the software, the QLF data was collected and stored onto a dedicated computerised data collection template using Microsoft Excel (Microsoft Corporation, Redmond, Washington, U.S.A).

2.7.2 Transverse Microradiography (TMR)

TMR is a destructive technique which requires the dental hard tissue samples to be cut into thin sections which are then analysed for mineral content. The technique is based on a direct comparison of the measurement of X-ray absorption by the tooth section and an appropriate standard (Joshi *et al.* 2016).

The enamel samples were placed onto circular ceramic discs using Greenstick impression compound (Kerr Corporation, California, U.S.A) with the exposed surface of the enamel sample facing up on the superficial aspect of the ceramic discs (Figure 2.24). The enamel samples were into approximately 700µm (thickness) sectioned using the WELL Precision Diamond Wire saw (Figure 2.13). The enamel samples were cut so that each section included both the exposed window and protect margins of the sample.

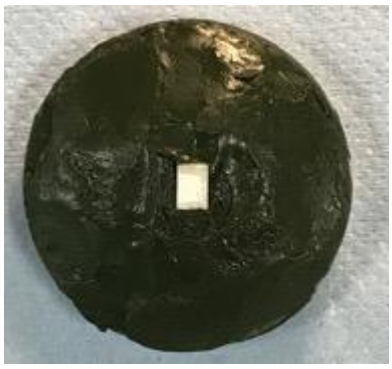


Figure 2.24: Enamel sample placed within greenstick compound on ceramic disc ready for sectioning

The sections were placed on top of 11mm custom made brass anvils and fixated onto the surface of the anvil using a combination of nail polish (Max Factor Red, Proctor and Gambler, Weybridge, U.K.) and acetone which acted as an adhesive for the samples to remain fixated on the brass anvils. The samples were carefully and delicately positioned on the brass anvil to ensure that there was no nail polish between the section and brass anvil. The brass anvils were then left to air dry for a minimum of 12 hours.

Figure 2.25: Enamel sections placed on brass anvils with nail polish and acetone acting as an adhesive

Once dried, the brass anvil was secured onto the top plate of the polisher using the screw in fixture. The sections were then polished by delicately moving the top plate with brass anvil fixated in a circular motion and the enamel sections were polished on the custom Diamond polishing disc using custom ball bearings, initially using 11.25mm diameter ball bearings which polished the section down to 250µm thickness (Figure 2.26). During the polishing process, cool water was applied to the diamond polishing disc surface to preserve the enamel section and prevent damage caused by the polishing procedure. Once the sectioned were polished to 250µm thickness the brass anvil was unscrewed, and the enamel sections were removed from the brass anvil using acetone and re-positioned on the brass anvil with the non-polished side facing upwards on this occasion. The process of placing and fixating the sections was the same as described above. On this occasion, custom ball bearings of 11.08mm thickness were used which polished the section to approximately 80-100µm which is the required thickness for TMR analysis.

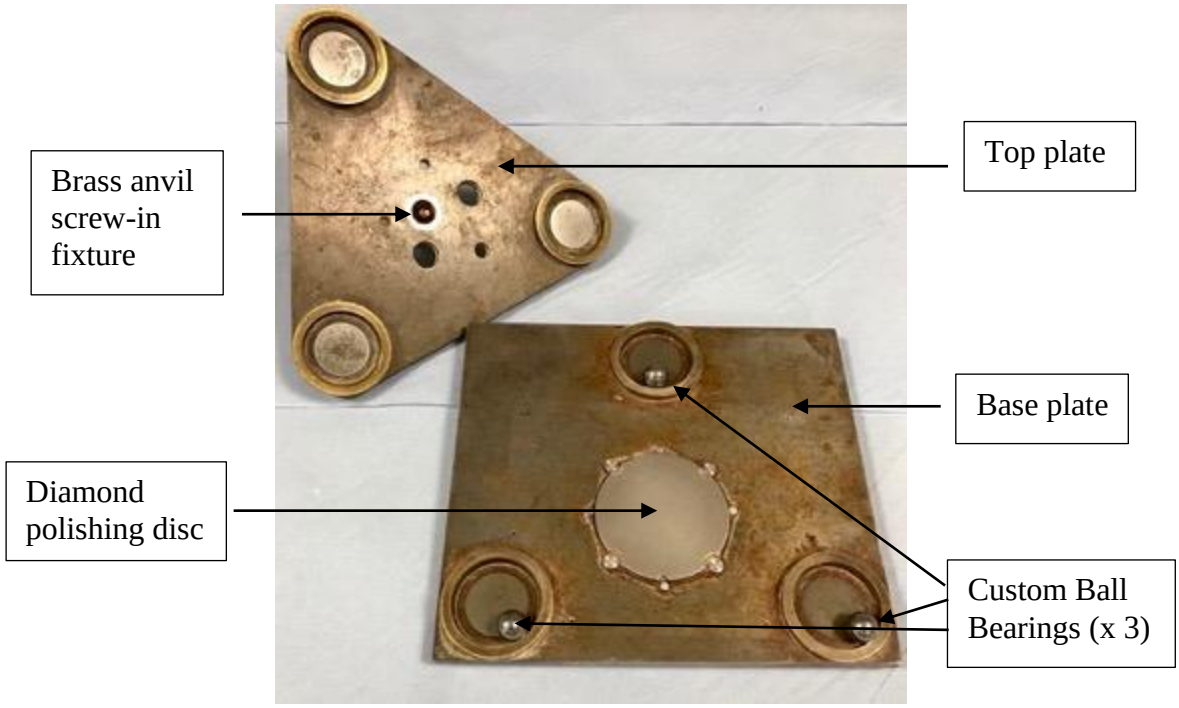


Figure 2.26: Set up apparatus for polishing TMR enamel sections

A digital micrometer (Mitutoyo No 293-766-30, Mitutoyo Corporation, Japan) was used to confirm sections were polished to the required thickness (approximately 80-100µm).

Figure 2.27 Digital Micrometer

Custom acetate template was cut with 3 rectangular windows cut as shown in Figure 2.28A. The top and bottom windows were used for placing the enamel sections whilst the middle window was for placement of the aluminium step wedge for calibration for the TMR analysis. The sections were placed on the acetate template (Figure 2.28) using double-sided adhesive tape (3M United Kingdom PLC, Berkshire, United Kingdom) with the exposed enamel (with lesion) sitting over the empty space and covered with thin film.

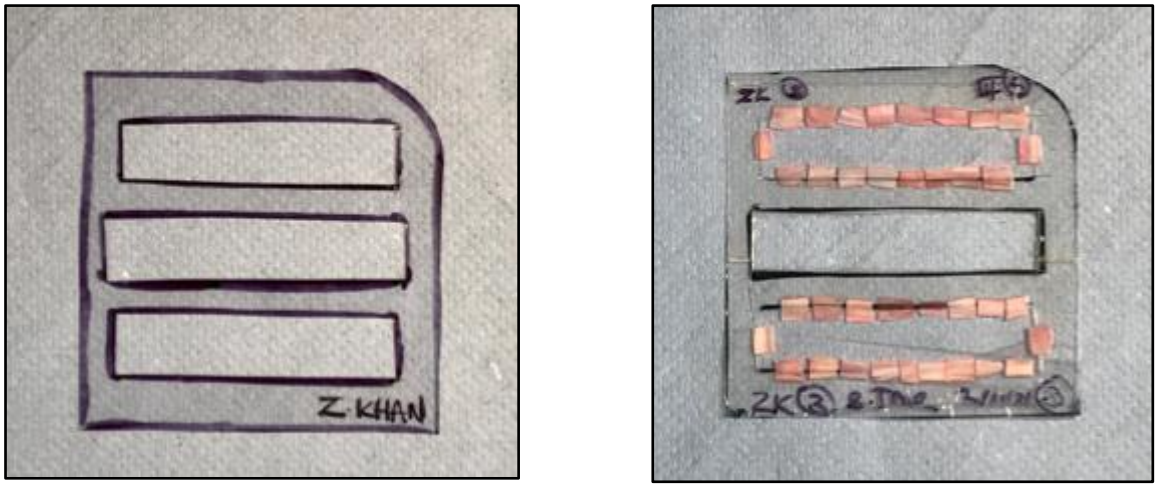


Figure 2.28: A -Acetate template and B - with sectioned placed on for TMR

The samples within the acetate template are then placed on a high-resolution radiographic plate (HTA Enterprises, California, United States of America) with the sections touching the radiographic plate and then on top of an aluminium calibration step-wedge and then irradiated with monochromatic x-rays. The emulsion layer of the radiographic plate was facing the x-ray source. The plate was then exposed to an x-ray source using the PW3830 x-ray generator (Figure 2.29)

Figure 2.29: PW3830 x-ray generator

The X-ray exposure time is set to 12 minutes with the settings at 20kV and 20mA (Garry *et al.* 2015). Once the x-ray has been generated, the x-ray image needs to be converted from a latent image to a visible image which is then subsequently used for

TMR analysis. The process for converting the image into a visible image is done using a developer (EMS Kodak D-19 Replacement, Electron Microscopy Sciences, Hatfield, United States of America) (Figure 2.30) and Fixer (Ilford Rapid Fixer, Harman Technology Ltd, Knutsford, Cheshire, United Kingdom) (Figure 2.30) in a dark room. Once the X-ray plate has been thoroughly rinsed with distilled deionised water and left to dry, it can then be analysed using the TMR software.

Figure 2.30 Developer and Fixer for converting latent to visible radiographic image

2.8 Statistical Analysis

For all statistical analysis, the significance level was set at □ = 0.05. All statistical analyses were performed using SPSS version 27 (SPSS Inc, Chicago, Illinois, USA). As per the study methodology, each of the 5 groups had 15 sound samples and 15 samples with demineralised lesions at baseline. For the sound samples, differences in mineral loss were determined using one-way ANOVA and multiple comparisons between the different groups was determined with post hoc Tukey Test. For the demineralised samples, statistical analyses were conducted using ANCOVA and Estimated Means Testing with Sidak adjustment.

2.9 Sample Size calculation

Previous work (Komarov and Higham 2007) has given an estimate of the mean ΔZ from exposure to Coca-Cola to be 3991.8 with a standard deviation of 434.6. Using this estimate of standard deviation, a sample size of 12 per group would allow detection of a difference in mean ΔZ of 600 between drinks, with 80% power and α=0.05. To

consider potential loss/damage of samples, a final number of 15 per group was used in this study (15 sound and 15 demineralised samples per group).

Chapter 3 Development of the Oral Health Cycling Model

3.1 Introduction

A model is a process which simulates, or relates to, some real-world phenomenon of interest; by studying models, the researcher hopes to derive information about that real-world phenomenon (Proskin 1992). Due to the ethical limitations with in vivo research, it may not always be possible to conduct a research study on human participants. Other limitations can be due to logistical or economic barriers. This opens the opportunity to conduct *in vitro* research which allows the researcher to circumnavigate any hindering issues that may prevent an in vivo or in situ study. In this study, a cycling model simulates the real-world conditions of the oral environment.

3.2 Background

In vitro research provides a bridge for in vivo research. However, to make *in vitro* research as representative and realistic as possible, it is essential to simulate clinical conditions accurately and thereby increase the reliability and applicability of the study.

There have been several pH cycling models that have been described and accepted within the scientific community. The models alternate between demineralising and remineralising conditions, with the aim of having either a net demineralising, or alternatively, a net remineralisation effect. An example of a demineralising model which is commonly used is the Featherstone pH cycling model (Featherstone *et al.* 1990, Featherstone *et al.* 2011, Stookey *et al.* 2011). This model has been shown to correlate with findings from human clinical trials (Lu *et al.* 1985) and can be utilised to create a carious lesion. In contrast, pH cycling models have been described where there is a net remineralising effect. This would be to test investigative products to assess their respective ability to remineralise an early carious lesion, in addition to the naturally occurring remineralisation effect of human saliva. A common example of such a remineralising pH cycling model is the Lippert model (Lippert *et al.* 2009). This model has been shown to be reliable and has been validated for artificial caries lesions produced in both human and bovine enamel (Lippert *et al.* 2012).

3.3 Types of Models used in *in vitro* Demineralisation-Remineralisation studies

A search of the current literature confirms the vast array of studies on dental cariology. These studies can either be *in situ, in vivo* or *in vitro*. Many of the studies that have been published are mechanistic studies with an aim of investigating caries

pathogenicity or assessing the effects of anti-caries products. Mechanistic studies on demineralisation-remineralisation aim to investigate both the molecular and physiological mechanism by which substances exert their effects on teeth (Yu *et al.* 2017).

Yu *et al.* (2017) describes the types of mechanistic studies in recent publications, which can be classified into *in situ, in vivo* or *in vitro*. The authors further expand to demonstrate that demineralisation-remineralisation studies employ either a chemical model or a biofilm model. Chemical models involve acidic challenges with the aim to replicate the environment of the oral cavity on a chemical level rather than a biological level (Skucha-Nowak *et al.* 2015). There are many advantages of using a chemical model for cariology research, such as simplicity of the study, low cost, reproducibility, and stability of the experiments, however, chemical models do not take into account the microbiological aspect of caries pathogenicity (Yu *et al.* 2017). *In vitro* experiments are the most commonly applied method in dental research and have a key advantage in the ability to conduct single carriable experiments under highly controlled conditions (White 1995).

3.4 Automated pH cycling models

pH cycling refers to *in vitro* experimental protocols including the exposure of substrates (enamel or dentine) to combinations of demineralisation and remineralisation (White 1995). The pH cycling model is a type of a chemical model used *in vitro* caries research. First described in 1982 by ten Cate and Duijsters, the pH cycling model aims to simulate the pH fluctuations *in vitro*. The model is based upon demineralising-remineralising alternations, with a pH neutral environment which is periodically interrupted by acid challenges.

Since the genesis of pH cycling protocols by ten Cate and Duijsters (1982), *in vitro* pH cycling models have been adapted to allow for automated cycling techniques. A number of automated cycling techniques have been described in the literature (Robinson *et al.* 1992, Almqvist and Lagerlöf 1993, Kirkham et al 1994).

Almqvist *et al.* (1990) applied a continuous delivery system to provide control of multiple pH cycles to investigate the effects of topical fluoride treatments (Almqvist *et*

al. 1990. Almqvist and Lagerlöf 1993). Automated cycling models have been employed to examine caries lesion progression under pH cycling conditions (Robinson *et al.* 1992) as well as to investigate the effects of various cycling ratios on lesion progression (Kirkham *et al.* 1994). The use of an automated cycling model allows the researcher to investigate factors which contribute to caries lesion progression, or alternatively to assess a variety of different products/substances to promote remineralisation of a caries lesion.

There are a number of advantages of automated pH cycling models, such as ease of use, logistical benefits, allowing for multiple studies to be conducted simultaneously and smaller sample size required (Yu *et al.* 2017). Automated cycling adjuncts are now available to allow the researcher to conduct a study with the aid of an automated robot. A robot is an automated machine which conducts various cycling protocols under the precise prescription of a dedicated bespoke computer software.

In this study, we used the Zinsser LISSY analytic cycling robot (Zinsser Analytic GmBH, Eschborn, Hessen, Germany) to conduct and develop this novel cycling model. Zinsser LISSY can run simultaneous cycling experiments on up to 6 different groups. As described in chapter 2 (2.5.1.9), the enamel samples are placed onto centrifuge tubes which are subsequently placed onto the bespoke rack to conduct the cycling protocol on Zinsser LISSY. Each tube with experimental enamel samples is positioned on the integrated robotic arm to allow the custom designed application to be conducted.

Zinsser LISSY is programmed using a dedicated computer software (WINLissy, Zinsser Analytic, GmBH, Eschborn, Hessen Germany) to set parameters. The software is programmed to run a bespoke cycling protocol, and this includes setting a number of cycling parameters, such as total number of cycles, duration, and frequency of immersions within different solutions and degree of agitation.

3.5 Rationale for the development of a novel cycling model

The current cycling models that have been described in the literature allow for the assessment of caries lesion progression and to test the efficacy of products/substances aimed to promote remineralisation of demineralised dental

substrate (enamel or dentine). In the current literature, a pH cycling model which accurately mirrors oral cavity conditions and encompasses all of the challenges faced on a daily basis has not been described. These challenges include exposure to a fluoride solution (which represents the introduction of fluoride ions to the dental hard tissue substrates with the use of a fluoride toothpaste during toothbrushing), a demineralising effect and drop in pH during the consumption of a meal and resting period in saliva between meals promoting remineralisation due to the natural buffering capacity of saliva (Lagerlof *et al.* 1984).

We utilised the novel cycling model to study the effects of energy and sports drinks on bovine enamel assessing the demineralising and erosive potential of the investigative drinks. The authors conducted a direct comparison of the test drinks with positive and negative controls. However, in order to accurately test these drinks and ensure that the results are as representative of real-world phenomenon and generalisable for human participants, we aimed to design a novel cycling model which simulates a 24-hour dietary cycle within the oral environment and takes into account the daily challenges encountered within the oral cavity and has a stable neutral effect on the dental substrates used. This would then allow for the assessment of the introduction of the test drinks on the enamel samples and confirm that any changes in mineralisation of the enamel samples are as a result of the test drinks and not caused by the cycling model itself.

3.6 Key Variables

In vitro cycling models allow for a number of key variables to be selected and adjusted as required. There are a number of key variables which were selected in the development of our novel cycling model and include:

- Saliva
- Fluoride
- pH fluctuations and challenges
- Frequency and Duration of pH fluctuations
- Positive and Negative Control

This model aims to closely represent the oral environment and as such, each of the above variables is discussed further in their respective sections below.

3.6.1 Saliva

The majority of salivary secretion (93% by volume) is secreted by the major salivary glands and the remaining (7% by volume) by the minor salivary glands (Llena-Puy 2006). Saliva is comprised of 99% water (Benn and Thomson 2014). The other components of saliva are sodium, potassium, calcium, magnesium, bicarbonate, phosphates, immunoglobulins, proteins, enzymes, mucins, urea, and ammonia (Humphrey and Williamson 2001, Whelton 2004). The normal pH of saliva is between 6 to 7, with a range from 5.3 (low flow) to 7.8 (peak flow) (Benn and Thomson 2014).

Saliva plays an important role in the oral cavity and serves numerous functions, such as lubrication (coats, protects against mechanical, thermal, chemical irritation, assists air flow, speech and swallowing), cleansing (moistening aids mastication, and clearing food), buffering (modulates pH of biofilm and buffering capacity of saliva), Ionic reserve (modulates demineralisation and remineralisation of teeth), digestion (enzymes in saliva begin the breakdown of starch and fat) and hydration (oral dehydration and dryness of the mouth, stimulates the desire to drink) (Humphrey and Williamson 2001, Whelton 2004, Thomson *et al.* 2011). The various components within saliva have differing functions and they also interact to enhance or inhibit other components' actions (Humphrey and Williamson, 2001).

Due to the natural composition of human saliva, it can introduce bacterial colonies and also contribute towards plaque development (Tang *et al.* 2003). This *in vitro* study did not involve the use of oral bacterium or plaque, therefore an appropriate substitute for human saliva was required. In this study we used artificial saliva which plays the role of natural saliva as would be found in the oral environment.

The use of artificial saliva in dental research dates back to the 1931 (Souder and Sweeney 1931). Since then, numerous scientific studies have been conducted to further develop artificial saliva for use in dental research and many variations on artificial saliva models have been described in the literature (Greenwood *et al.* 1937, Muhler and Swenson 1974, Darvell 1978, El Mallakh and Sarkar 1990, Gal *et al.* 2001). Due to the effect of different variables on salivary composition, it is not possible

to create a single model of artificial saliva that is universally applicable (Pytko-Polonczyk *et al.* 2017). In this study, the use of an artificial saliva was intended to serve similar functions of natural saliva, in particular, to act in a buffering capacity, artificial saliva was incorporated into the cycling model to replicate rest periods between meals and over-night. The formula for the artificial saliva used in this study is detailed in Table 3.1.

Chemical	Weight (g)
Methylhydroxybenzoate	2.000
Sodium carboxymethylcellulose	10.000
Potassium chloride	0.625
Magnesium chloride hexahydrate	0.059
Potassium hydrogen orthophosphate	0.804
Potassium dihydrogen orthophosphate	0.326
Calcium chloride dihydrate	0.166

Table 3. 1 Composition of artificial saliva used in this study

The artificial saliva is made up using deionised water to 1 litre with the pH adjusted to 7.2 using concentrated Potassium Hydroxide or 1M Hydrochloric acid.

3.6.2 pH Fluctuations within the Oral Cavity

The oral cavity undergoes fluctuations in pH, and this leads to alternating states of demineralisation and remineralisation. Of particular importance is the plaque pH. As has been shown by Stephan (1944), following the consumption of sugars, such as sucrose, there is a rapid drop in the plaque pH, and this creates a cariogenic environment.

The pH drops below the critical pH of 5.5 and thus a demineralising environment is created (Figure 3.1). The natural buffering characteristics of saliva gradually leads to a rise and recovery in the pH within the oral environment to its original value (which is above pH 7.0) and this process occurs over a period of 20-30 minutes to several hours (Stephan 1944).

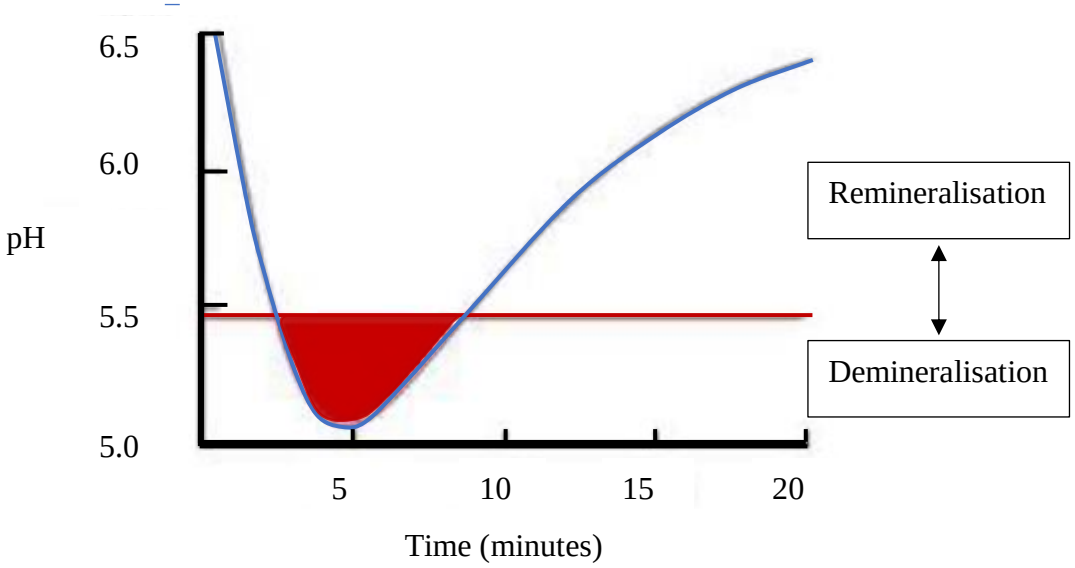


Figure 3. 1 Stephan Curve (Adapted from Stephan 1944)

With each carbohydrate intake, the pH drops below its critical value and frequent intake of carbohydrates with the consumption of food stuff leads to successive drops in pH and resultant pH fluctuations. Once consumed, the fermentable substrates of carbohydrate are metabolised by the plaque microflora which leads to acid production (Bibby and Krobiccka 1984) and subsequently, the pH drops below its critical value. Figure 3.2 illustrations the pH fluctuations with frequent intake of carbohydrates.

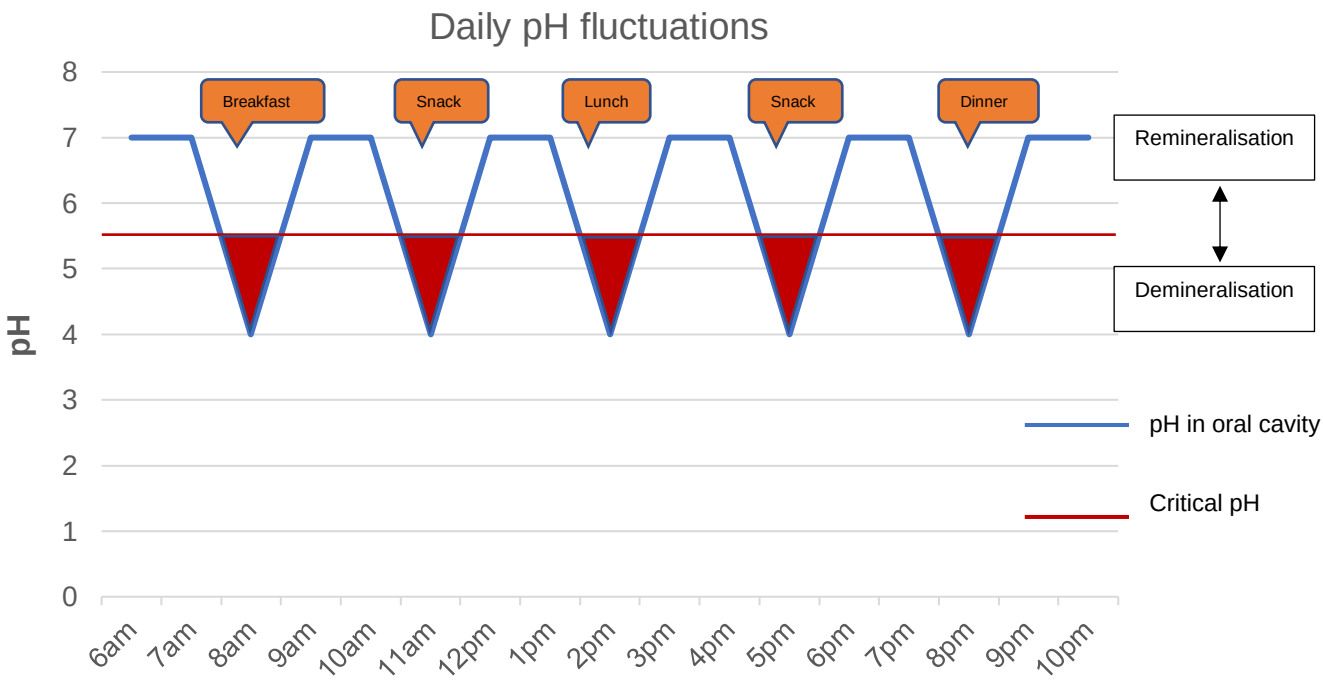


Figure 3. 2 Schematic illustration of daily pH fluctuations

Several studies have been conducted to investigate caries progression using a pH cycle and various protocols have been designed (Herkstorter *et al.* 1991, ten Cate *et al.* 1995, White 1987, ten Cate and Duijsters 1982) to study demineralisation at different pH solutions. These studies however were not designed to replicate a standard 24-hour dietary cycle, simulating pH fluctuations, but rather they were designed to study the process of demineralisation and factors affecting cariogenecity.

In the newly developed model described in this study, we aimed to replicate the pH fluctuations encountered over a 24-hour period, with each exposure to the demineralising meal solution replicating the consumption of a standard meal and the subsequent fall in pH. A standard meal solution was created to replicate the demineralising effect during the consumption of a meal. Table 3.2 details the composition of the demineralising meal solution used in this study:

Chemical	**Amount**
Potassium dihydrogen orthophosphate	0.299g
Calcium Chloride Aqueous Volumetric Solution Grade	2.2ml
Glacial Acetic acid (HAC) AR grade	2.85ml
Distilled De-ionised water	250ml
Potassium Hydroxide	Adjusted according to desired pH
Sodium Fluoride solution	0.5ml (500µl)

Table 3. 2 Composition of Demineralising Meal Solution

Following the addition of the above ingredients, the solution is made up to 1 litre using deionised distilled water. The purpose of the demineralising meal solution was to simulate the drop in pH during meal consumption and importantly, the use of a demineralising meal solution should not result in demineralisation during the cycling model. In this study, a standard pH 4.5 demineralising meal solution (Table 3.3) was initially used to simulate the drop in pH to below the critical pH.

3.6.3 Fluoride and Oral Health

The link between natural fluoride, adjusted fluoride levels in drinking wate and reduced dental caries prevalence was made in the early 1940s (Dean 1942) and since then

there has been much on-going research into fluoride and caries prevention. However, it was only in the second half of the 20th century with the successful incorporation of fluoride that toothpaste acquired a therapeutic anti-caries effect (Mullane *et al.* 2016). Over the years various fluoride compounds have been added to toothpaste such as sodium fluoride, acidulated phosphate fluoride, stannous fluoride sodium monofluorophosphate and amine fluoride (Lippert 2013).

Several systematic reviews have reported on the effectiveness of fluoride toothpaste at preventing dental caries (Marinho *et al.* 2003a, Twetman *et al.* 2003, Twetman 2009, Wright *et al.* 2014) and the study by Goldman *et al.* l (2008) confirmed that fluoride toothpaste is now the most widely used method of maintaining a constant low level of fluoride within the oral cavity. In the U.K. there has been a significant reduction in the prevalence of dental caries in children over the past few decades (NHS Digital 2015) and this has largely been attributed to the introduction of fluoride toothpastes in the early 1970s (Pitts *et al.* 2017). Walsh *et al.* (2010) conducted a systematic review into the effectiveness of different concentrations of fluoride toothpaste and the review confirmed the caries-preventative benefits of using fluoride toothpaste compared to a placebo treatment in permanent teeth when at a concentration of 1000ppm or above.

In the U.K. the Department of Health and Social Care, in combination with Public Health England, NHS England and other health authorities, have set guidelines on a U.K. wide approach to the prevention of oral diseases (Delivering Better Oral Health 2021). The Delivering Better Oral Health publication is an evidenced based toolkit developed to support dental teams in improving their patient's oral and general health. The Delivering Better Oral Health toolkit recommends the use of a family or standard fluoride toothpaste at 1350-1500 part per million (ppm) for children, adolescents, and adults, although for very young children where swallowing is limited, a lower amount of fluoride within the toothpaste is suggested (minimum of 1000ppm) (SIGN 2014). The toolkit advises further on the importance of the frequency of brushing, with a recommendation of a minimum of brushing twice daily.

Taking these recommendations into account, the novel cycling model that has been developed in this study has incorporated the effect of brushing twice daily into our cycle with respect to the introduction of fluoride containing solutions. Although this

model did not involve any mechanical toothbrushing involving the use of bristles to clean the tooth surface, it does however involve the use of a fluoride solution which mimics the fluoride ion exposure to teeth during brushing.

3.6.4 Frequency and Duration of pH fluctuations

The novel cycling model developed in this study has been based upon the societal norms of an average of three meals a day and as such the model includes 3 separate immersions within a demineralising meal solution, which would replicate the drop in pH during the consumption of a meal. The 3 separate occasions represent meals at breakfast, lunch, and dinner.

With respect to the duration of a meal, the novel cycling model is based upon the work of Stephan (1944), in particular on the Stephan curve which demonstrates the demineralising effect to last for 20-30 minutes following the intake of a carbohydrate containing meal. Therefore, the duration of exposure to a demineralising meal solution was set to 30 minutes. This would be repeated 3 time per cycle to reflect 3 daily meal consumption.

3.6.5 Positive and negative control

To check methodology, the inclusion of a standard acid solution is desirable (Shellis *et al.* 2011). The positive control is a treatment which with a well-known effect and conversely a negative control is a treatment which is not expected to have any effect. In this study, Coca-Cola was chosen as the positive control. Energy and sports drinks are acidic in nature and therefore, the selection of an acidic drink such as Coca-Cola was an appropriate choice. The literature describes a vast array of studies that have been conducted to investigate the effects of Coca-Cola on dental hard tissues (Maupomé *et al.* 1998, Kitchens and Owens 2007, Yuan 2016). The negative control was deionised distilled water. During the series of pilot studies to develop the cycling model, once the robustness of the automated cycling platform was established, only a negative control group was used to confirm that the model does not lead to any demineralisation on the enamel samples.

3.7 Pilot studies

This section details a series of *in vitro* pilot studies to develop the Oral Health cycling model.

3.7.1 Pilot Study 1 (P1)

The first pilot study was conducted to assess the accuracy and efficiency of the Zinsser LISSY cycling robot as well as assessing the cycling protocol overall.

This pilot included 3 groups:

i) Negative Control: Deionised distilled water
ii) Test drink: Red Bull Energy Drink
iii) Test drink: Monster Energy drink

The protocol was programmed to include two minutes of exposure to 1450ppm Fluoride (F) solution at the start of each cycle. This represented the exposure to fluoride ions within the oral cavity during toothbrushing, once at the start and once at the end of each cycle to mimic brushing with fluoride toothpaste twice (in accordance with the Delivering Better Oral Health Toolkit). The samples were washed with immersions in distilled deionised water between exposures to the different solutions to ensure that there was no cross contamination between the different solutions. Following the fluoride solution exposure, the samples were exposed to a demineralising meal solution (Meal solution) at pH 4.5 to replicate the drop in pH during the consumption of a meal. Thereafter, the samples were exposed to either a challenge or control medium: Red Bull energy drink, Monster energy drink or deionised distilled water respectively.

The cycling protocol was established and programmed into the dedicated computer software to accurately set the parameters for the cycling process. One of the aims of the pilot study was to assess the effects of the test drinks and negative control on bovine enamel. It was expected that the negative control group samples should not undergo demineralisation. This was required to confirm the novel cycling model itself does not result in any enamel demineralisation and therefore the negative control group samples should remain sound throughout the cycling process. The primary outcome measure was enamel mineral loss using TMR and QLF techniques. The

cycle was conducted for 14 complete cycles to represent 14 days. The cycling protocol used in P1 is shown in Table 3.3.

Medium	Duration (minutes)
Start	
1450ppm F solution	2
Wash	1
Meal solution	15
Wash	1
Challenge/Control	10
Wash	1
Meal solution	15
Wash	1
Challenge/Control	10
Wash	1
Meal solution	15
Wash	1
Challenge/Control	10
Wash	1
1450ppm F solution	2
End	

Table 3.3 P1 cycling protocol

P1 Results

After 14 full cycles were conducted, the samples were removed, washed under distilled water, and left to air dry.

The QLF and TMR analysis showed mineral loss (erosive lesions) for the Red Bull samples, Monster samples and surprisingly also for the negative control group samples. Although this pilot study confirmed the accuracy and efficiency of the Zinsser cycling robot for conducting automated pH cycling, it did not result in the negative control group samples remaining sound throughout the cycling model. This would indicate that the cycling model has a net demineralising effect and adjustments are required to ensure the negative control samples do not undergo demineralisation.

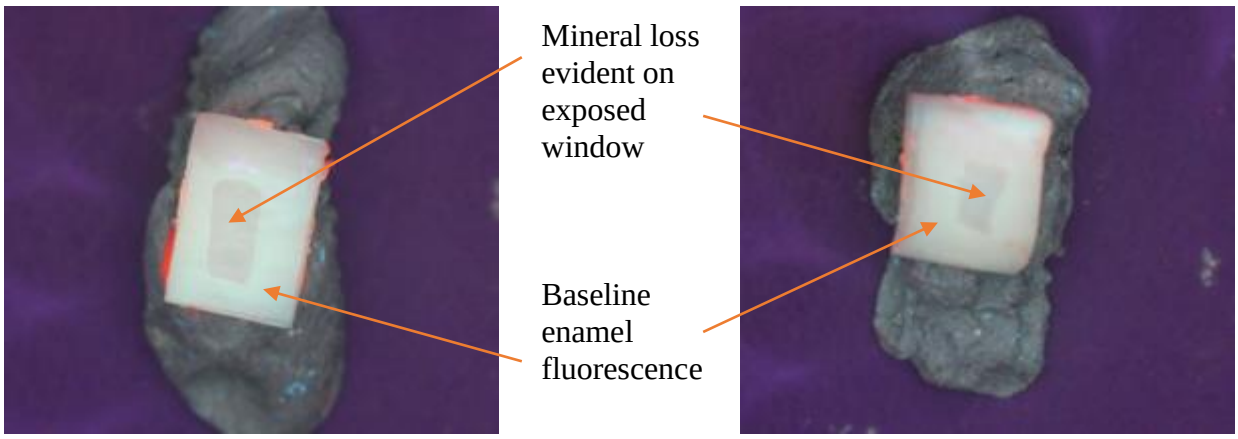


Figure 3.3 QLF images of negative control samples (A,B) with demineralised lesions on exposed window

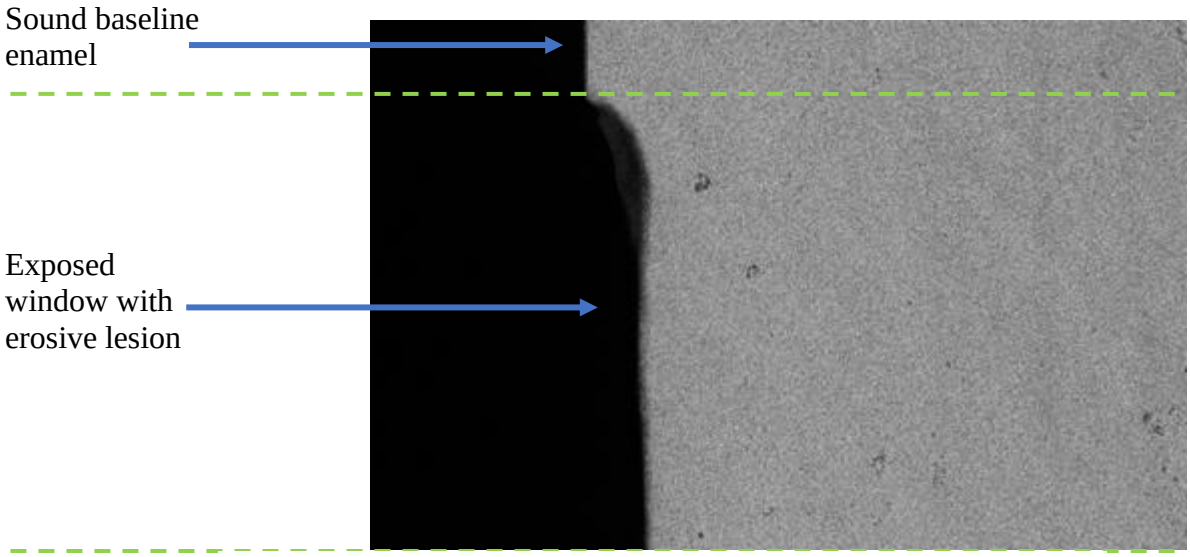


Figure 3.4 TMR image of negative control sample demonstrating erosion lesion following completion of P1

The results from P1 confirmed that the cycling protocol required modifications to ensure the negative control samples remained sound throughout the cycling process. P1 confirmed that cycling robot accurately performed all tasks however, the cycling model produced demineralised lesions in the control group samples. Therefore, this indicated that the cycling protocol produced a net demineralisation effect and needed modifications to ensure the negative control group samples did not undergo demineralisation. After P1, all further pilot studies were conducted with a focus only on the negative control group samples to produce a cycling model which exposes the samples to the various pH challenges without the samples undergoing enamel mineral loss.

3.7.2 Pilot Study 2 (P2)

There were a number of modifications made to the cycling protocol in P2. These included:

- Fluoride solution (F) concentration changed to 228ppm. This was to represent the dilution of fluoride toothpaste in the oral cavity and the diluted concentration to 228ppm as has been shown by Vogel *et al.* (2004).
- Artificial Saliva (A.S.) used as the negative control group solution.
- Incorporating rest periods between immersions.

The cycling protocol used in P2 is shown in table 3.4.

Medium	Duration (minutes)
Start of cycle	
228ppm F solution	2
Wash	1
Meal solution	30
Wash	1
Control (A.S.)	10
Wash	1
Rest period	30
Meal solution	30
Wash	1
Control (A.S.)	10
Wash	1
Rest period	30
Meal solution	30
Wash	1
Control (A.S.)	10
Wash	1
228ppm F solution	2
Rest period	30

Table 3. 4 P2 cycling protocol

P2 Results

After 14 full cycles were completed, the samples were removed, rinsed with deionised distilled water and left to air dry. QLF and TMR analysis was conducted on the enamel samples used in P2. QLF analysis confirmed mineral loss in all of the samples (Figure 3.4). TMR analysis showed the samples had erosive lesions, as was the case in P1.

Figure 3.5 shows the TMR images demonstrating the effect on the exposed enamel in comparison to the preserved baseline sound enamel. The cycling protocol at this stage was having a net demineralising (erosive) effect on the enamel samples.

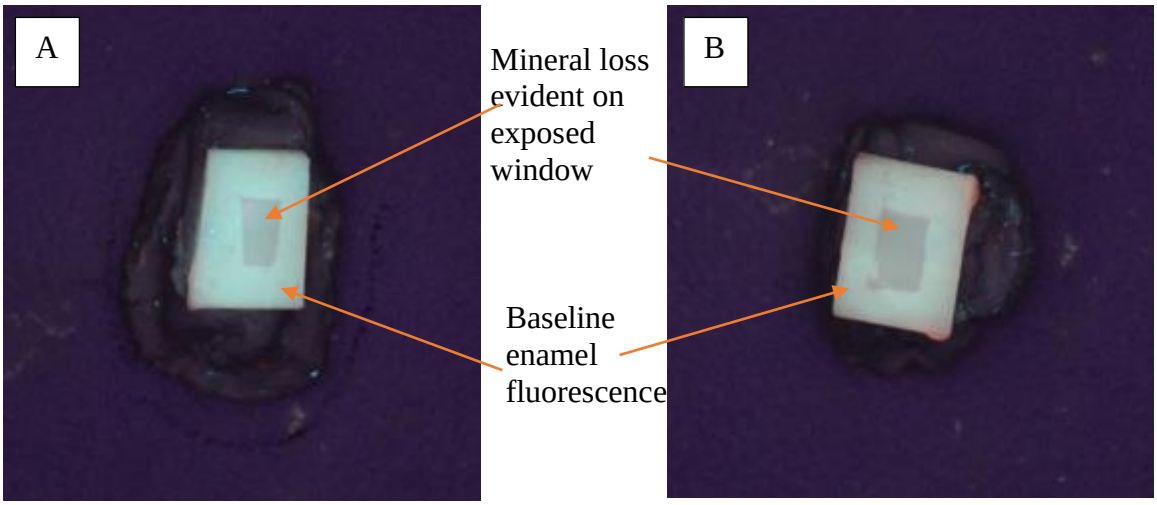


Figure 3.5 QLF images of samples (A,B) in P3 with demineralisation evident on the exposed window

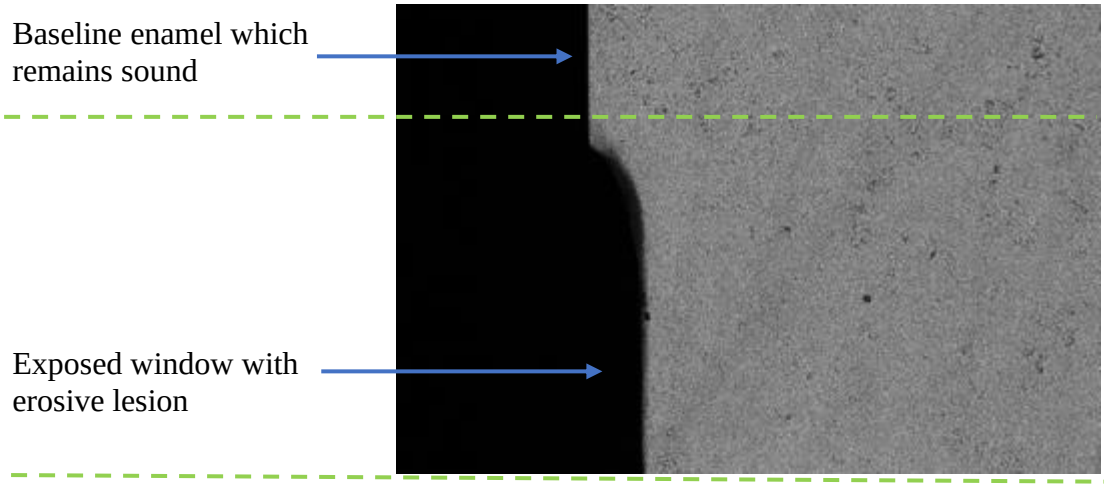


Figure 3.6 TMR image of sample after P2 demonstrating erosion lesion

3.7.3 Pilot Study 3 (P3)

P1 and P2 results showed the overall cycling protocol was resulting in a net demineralising effect on the negative control group samples. Further analysis and appraisal of the cycling protocol parameters was carried out to gauge what adjustments were required in this model. Following a detailed evaluation of the cycling protocol and further literature review, it was agreed that the overall duration of the previous cycle was too short and not fully representative of a 24-hour oral dietary cycle. With this in mind, the cycling protocol in P3 was modified with a few significant alterations. These adjustments included introducing time in artificial saliva at the

beginning of each cycle for 4 hours (to represent rest period during the night and before toothbrushing). This was based upon the study by Ablal *et al.* (2009) where samples were immersed in artificial saliva at the start of the cycling process. In addition to this, artificial saliva was also added between mealtimes and challenge medium. These changes increased the overall cycle time from 3 hours 40 minutes/cycle (P2) to 9 hours 16 minutes in P3, an overall increase in time of approximately 255% from the P2 cycling protocol. This change was introduced to increase the time within artificial saliva over the course of one complete cycle so that the buffering capacity of artificial saliva could be amplified. The cycling protocol used in P3 is shown in Table 3.5

Medium	Duration (minutes)
Start of cycle	
*Artificial saliva	240
Wash	1
228ppm F solution	2
Wash	1
Meal solution	30
Wash	1
*Artificial Saliva	60
Wash	1
Control medium	10
Wash	1
Meal solution	30
Wash	1
*Artificial Saliva	60
Wash	1
Control medium	10
Wash	1
Meal solution	30
Wash	1
*Artificial Saliva	60
Wash	1
Control medium	10
Wash	1
228ppm F solution	2
Wash	1
End of cycle	

*Introduction of rest period within Artificial saliva

Table 3. 5 P3 cycling protocol

P3 Results

TMR analysis for P3 confirmed that the samples had undergone subsurface demineralisation. Figures 3.7 and 3.8 are representative of the resultant subsurface demineralisation that the negative control samples had undergone in P3.

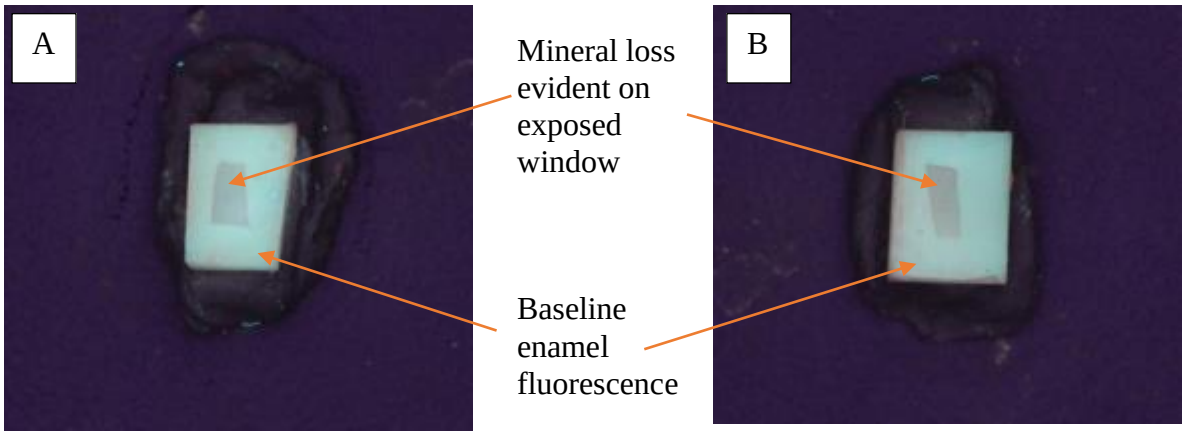


Figure 3.7 QLF images of samples (A,B) after P3 demonstrating demineralisation on the exposed window

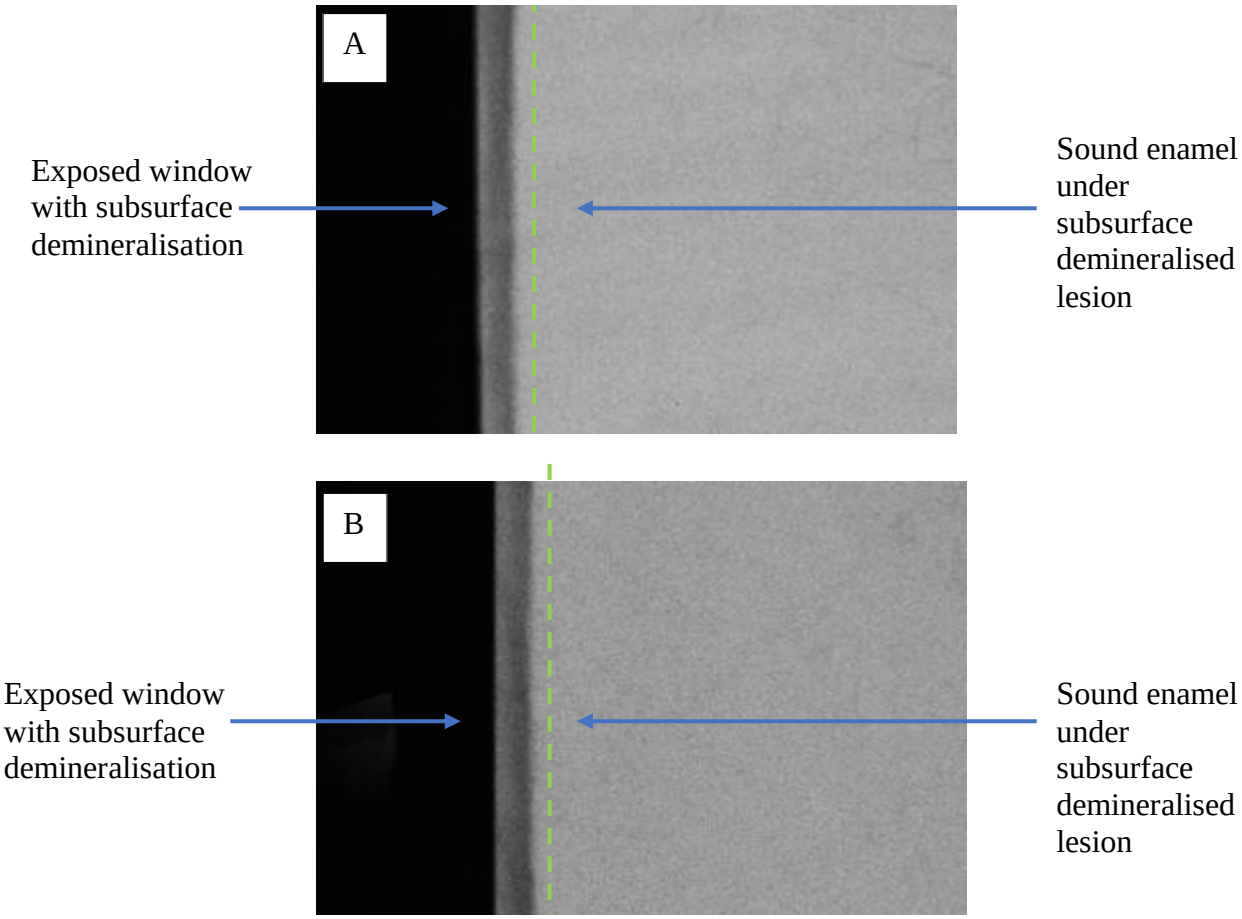


Figure 3.8 TMR images of samples (A,B) after P3 demonstrating subsurface demineralisation

The results from P3 demonstrated that although there no erosive lesions created under the cycling protocol, there was subsurface demineralisation on the negative control samples. Therefore, further modifications are required to the cycling protocol, and these are discussed in the next section.

3.7.4 Pilot Study 4 (P4)

The time selected for the demineralisation solution was 30 minutes, which is based Stephan's curve on the drop in plaque pH following consumption of sugars, and the subsequent return to a neutral pH with the buffering capacity of saliva. The duration of one complete cycle in P3 was approximately 556 minutes. After analysing the results and studying the cycling protocol, it was felt that the demineralisation time of 30 minutes (x 3 per cycle) was excessive considering the thickness of the samples and the length of the entire cycle. This would result in an overall excessive demineralising effect and is not representative of the proportional amount of demineralisation that would happen in an average 24-hour cycle within the oral cavity.

As a result, in P4, the significant change that was made was to proportionally reduce the time in demineralising solution based on a 24-hour cycle. Therefore, after calculating what would be the total demineralisation time (based on the Stephan's curve model) over 24 hours, the time in demineralising solution was proportionally reduced to 11 minutes to more accurately reflect a 24-hour cycle. The overall length of one cycle in P4 was 499 minutes and overall time in demineralising solution for each cycle was 33 minutes. In addition, the control medium was distilled deionised water.

The remainder of the cycling protocol was maintained as per the P3 cycling protocol. In P4, the cycling was conducted for 14 complete cycles. The cycling protocol used in P4 is shown in Table 3.6.

Medium	Duration
Start of cycle	
Artificial saliva	240
Wash	1
228ppm F solution	2
Wash	1
Meal solution	11*
Wash	1
Artificial Saliva	60
Wash	1
Control	10
Wash	1
Meal solution	11*
Wash	1
Artificial Saliva	60
Wash	1
Control	10
Wash	1
Meal solution	11*
Wash	1
Artificial Saliva	60
Wash	1
Control	10
Wash	1
228ppm F solution	2
Wash	1
End of cycle	

Table 3.6 P4 cycling protocol

*Proportional reduction to time in meal solution

P4 Results

After the conclusion of the 14 cycles, the samples were analysed using QLF and TMR to assess for mineral loss (ΔF and ΔZ respectively). QLF analysis demonstrated that all samples had mineral loss on the exposed central window (Figure 3.9). TMR

analysis confirmed that there were no erosive craters produced after P4, however, the samples all had evidence of subsurface demineralisation (Figure. 3.10). This was similar to the overall effects found after P3 but in P4, the degree of subsurface demineralisation was reduced. This indicated that the modifications made in P4 were appropriate and the degree of demineralisation was being reduced. Figures 3.9 and 3.10 demonstrate the QLF and TMR images of the samples after P4..

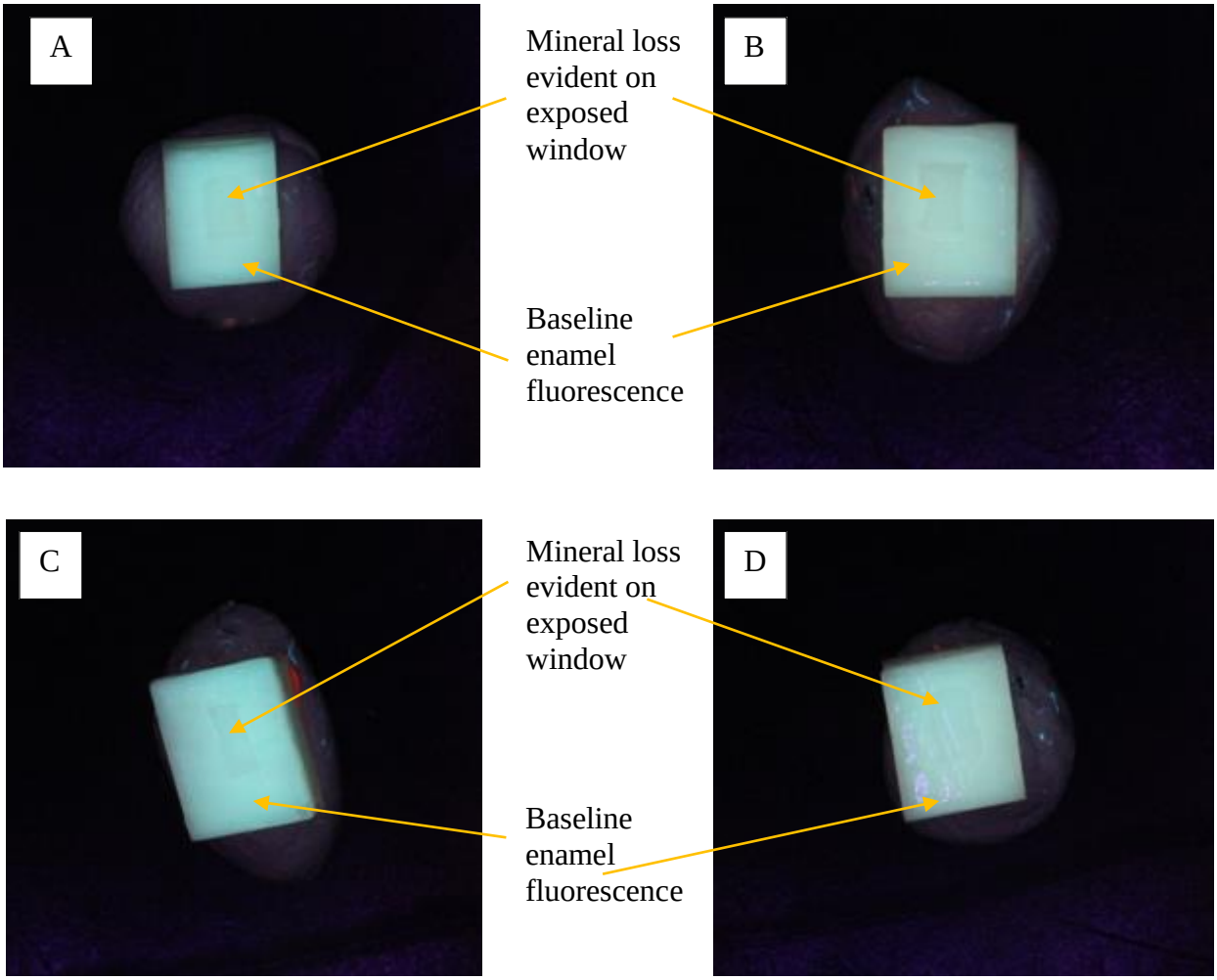


Figure 3.9 QLF images for samples (A, B, C, D) after P4

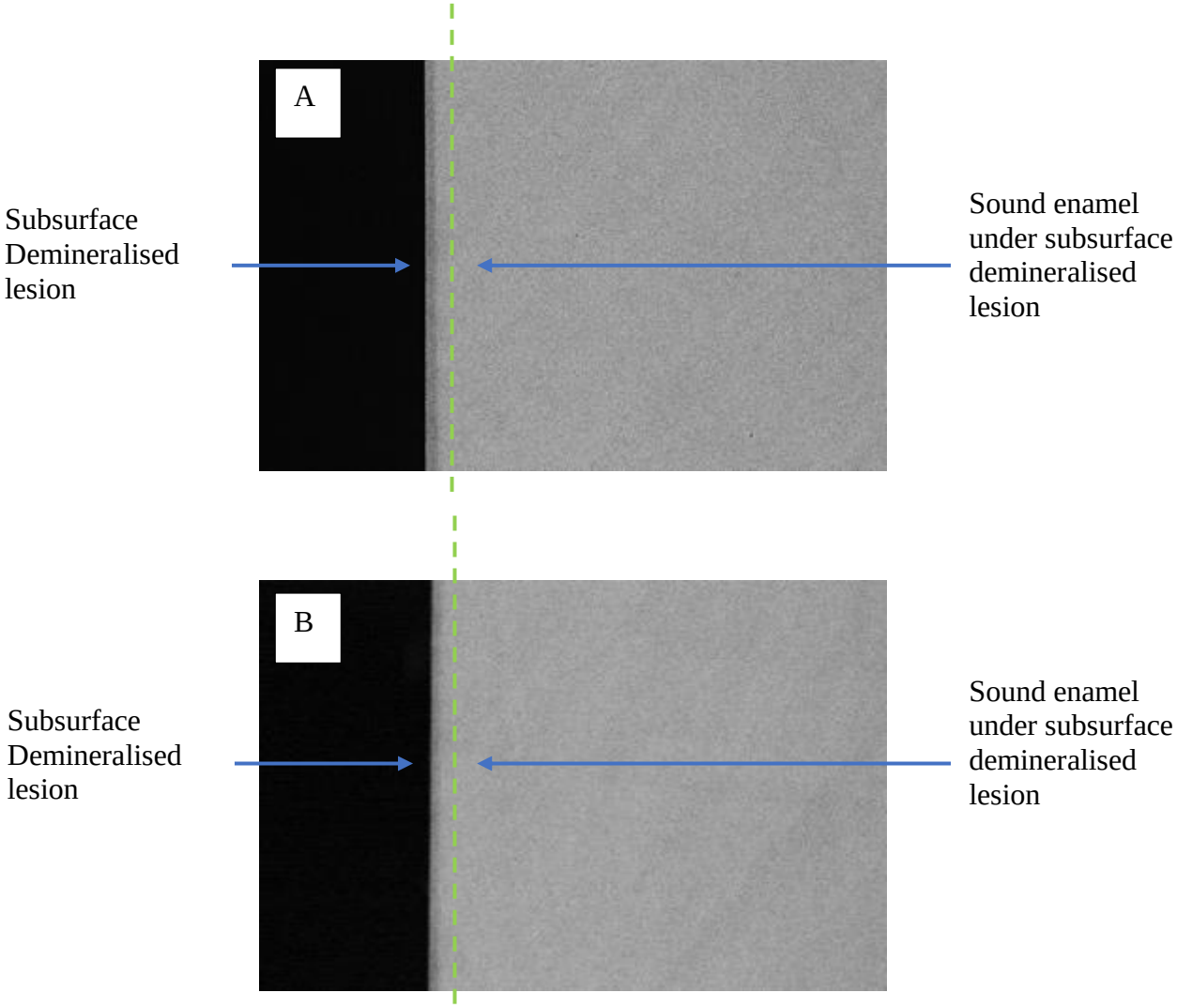


Figure 3.10 TMR images of samples after P4 (A,B) demonstrating subsurface demineralisation

3.7.5 Pilot Study 5 (P5)

P3 and P4 results showed that the novel cycling model had been developed to completely eliminate the creation of erosive crater lesions, however subsurface demineralisation was still being produced within the enamel samples. Although, the degree of subsurface demineralisation in P4 was significantly less than in P3, the overall effect was similar.

After further analysis and discussion, it was decided to further increase the overall time of the cycling protocol to make it more representative of a complete 24-hour cycle. In addition, the rest times within artificial saliva also needed adjustments to ensure a more realistic representation of rest periods between meals.

Therefore, in P5, the overall time for one complete cycle from increased from 499 minutes (P4) to 829 minutes. The time in artificial saliva at the beginning of the study was increased from 240 minutes to 480 minutes. This was based on previous work by Ablal *et al.* (2009) designed to replicate the “over-night” rest period within artificial saliva. In addition, the rest period between meals was increased with time in artificial saliva from 60 minutes to 90 minutes. The mealtime was maintained at 11 minutes as per the cycling protocol in P4.

These modifications to the cycling protocol were aimed to not only increase the time within artificial saliva to reflect a normal 24-hour dietary cycle, but also to increase the overall duration of each cycle to bring it closer to the target 24-hour cycle. As per previous pilot studies, the cycling was conducted on 14 consecutive occasions to mirror a 14-day time period. Table 3.7 demonstrates the cycling protocol used in P5.

Medium	Duration (minutes)
Start of cycle	
Artificial saliva	480
Wash	1
228ppm F solution	2
Wash	1
Meal solution	11
Wash	1
Artificial Saliva	90
Wash	1
Control	10
Wash	1
Meal solution	11
Wash	1
Artificial Saliva	90
Wash	1
Control	10
Wash	1
Meal solution	11
Wash	1
Artificial Saliva	90
Wash	1
Control	10
Wash	1
228ppm F solution	2
Wash	1
End of cycle	

Table 3.7 P5 cycling protocol

P5 results

As per previous pilot studies, P5 was conducted for 14 complete cycles and the samples were analysed using QLF (Delta F) and TMR (Delta Z) to assess mineral loss. QLF analysis did not detect any mineral loss (Figure 3.11). Further analysis for the primary outcome measured was conducted using the TMR technique. TMR analysis showed that the samples had once again undergone subsurface

demineralisation. Figures 3.12 demonstrates the typical subsurface demineralisation seen after P5.

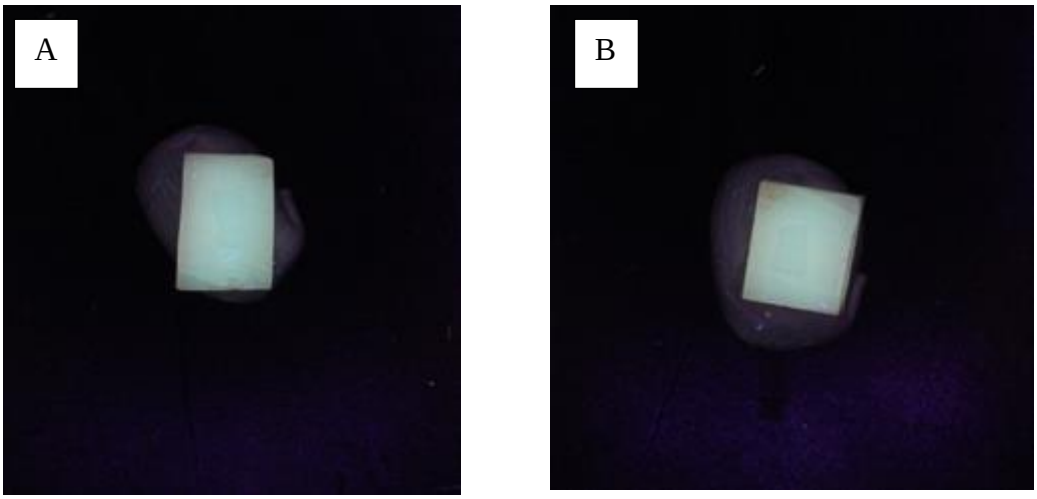


Figure 3.11 QLF images for samples (A,B) in P5. Mineral loss was not detected on any of the samples.

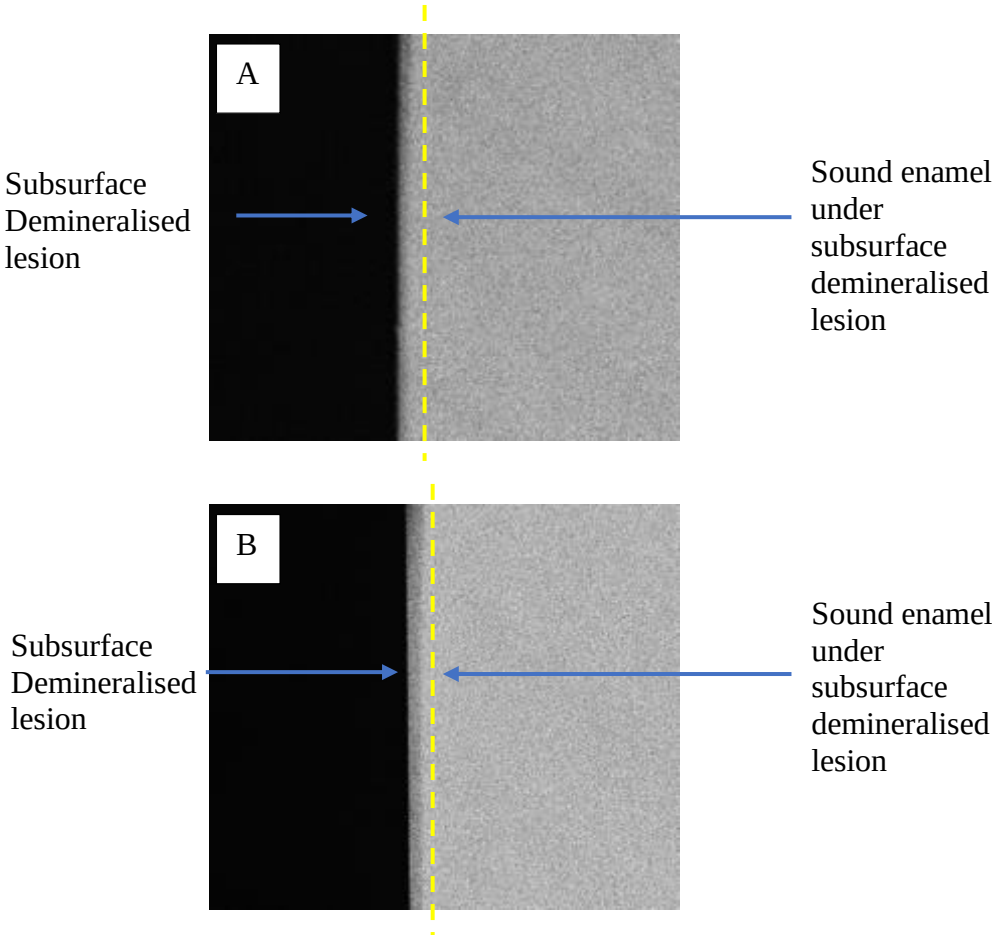


Figure 3.12 TMR images of samples (A,B) after P5 demonstrating subsurface demineralisation

Despite the increase in the overall duration of the cycling protocol and an increase of time within artificial saliva, the samples still had evidence of enamel mineral loss. The degree of subsurface demineralisation was however lower than the previous pilot studies. This once again confirmed that the cycling model was moving in the right direction, and it was felt that further adjustments would be required to confirm a novel cycling model for *in vitro* research.

3.7.6 Pilot study 6 (P6)

The protocol established in previous pilot studies was based on a 24-hour cycle with the overall duration of each cycle proportionally reduced. This was to maximise the efficiency of the *in vitro* model. The rationale behind the proportional reduction based on a 24-hour cycle was that if a 24-hour cycle could be proportionally reduced, it would lead to gains in terms of time and efficiency.

However, following further review and discussions, it was decided to conduct a full 24-hour cycle in an attempt to make the *in vitro* model fully representative of 24-hour cycle. It was anticipated that by further increasing the time in artificial saliva, this would lead to a further increase in the buffering capacity on the samples and thus not lead to any demineralisation of the negative control samples.

The modifications to the cycling protocol in P6 included increasing the overall duration of each cycle to 24 hours (1440 minutes). The time in artificial saliva at the beginning was maintained at 480 maintained (as per previously published study by Ablal *et al.* 2009). The time in artificial saliva between demineralising meal solution was increased from 90 minutes (P5) to 145 minutes and the time in demineralising meal solution was increased from 11 minutes (P5) to 20 minutes. This was to make the demineralising time more reflective of Stephan's curve. The cycling protocol used in P6 was shown in Table 3.18.

Medium	Duration
Start of cycle	
Artificial saliva	480
Wash	1
228ppm F solution	2
Wash	1
Meal solution	20
Wash	1
Artificial Saliva	145
Wash	1
Control	10
Wash	1
Artificial Saliva	145
Wash	1
Meal solution	20
Wash	1
Artificial Saliva	145
Wash	1
Control	10
Wash	1
Artificial Saliva	145
Wash	1
Meal solution	20
Wash	1
Artificial Saliva	145
Wash	1
Control	10
Wash	1
Artificial Saliva	145
Wash	1
228ppm F solution	2
Wash	1
End of cycle	

Table 3.8 P6 cycling protocol

P6 Results

After the completion of P6, the samples were analysed using QLF and TMR. QLF analysis confirmed there was no evidence of mineral loss on the exposed window of the samples (Figure 3.13). However, TMR analysis confirmed that there was mineral loss with subsurface demineralisation evident once again amongst the samples (Figure 3.14).

Despite the modifications made to the cycling protocol in P6, the overall effect of the cycling method was still a net demineralising effect. Figures 3.13 and 3.14 display QLF and TMR images for samples after P6.

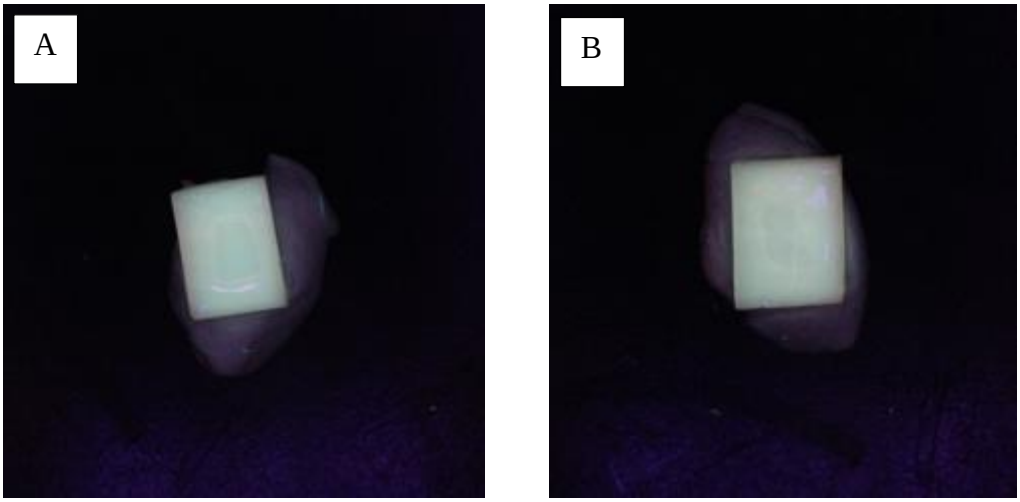


Figure 3.13 QLF images of samples (A,B) in P6. Mineral loss was not detected on any of the samples in P6.

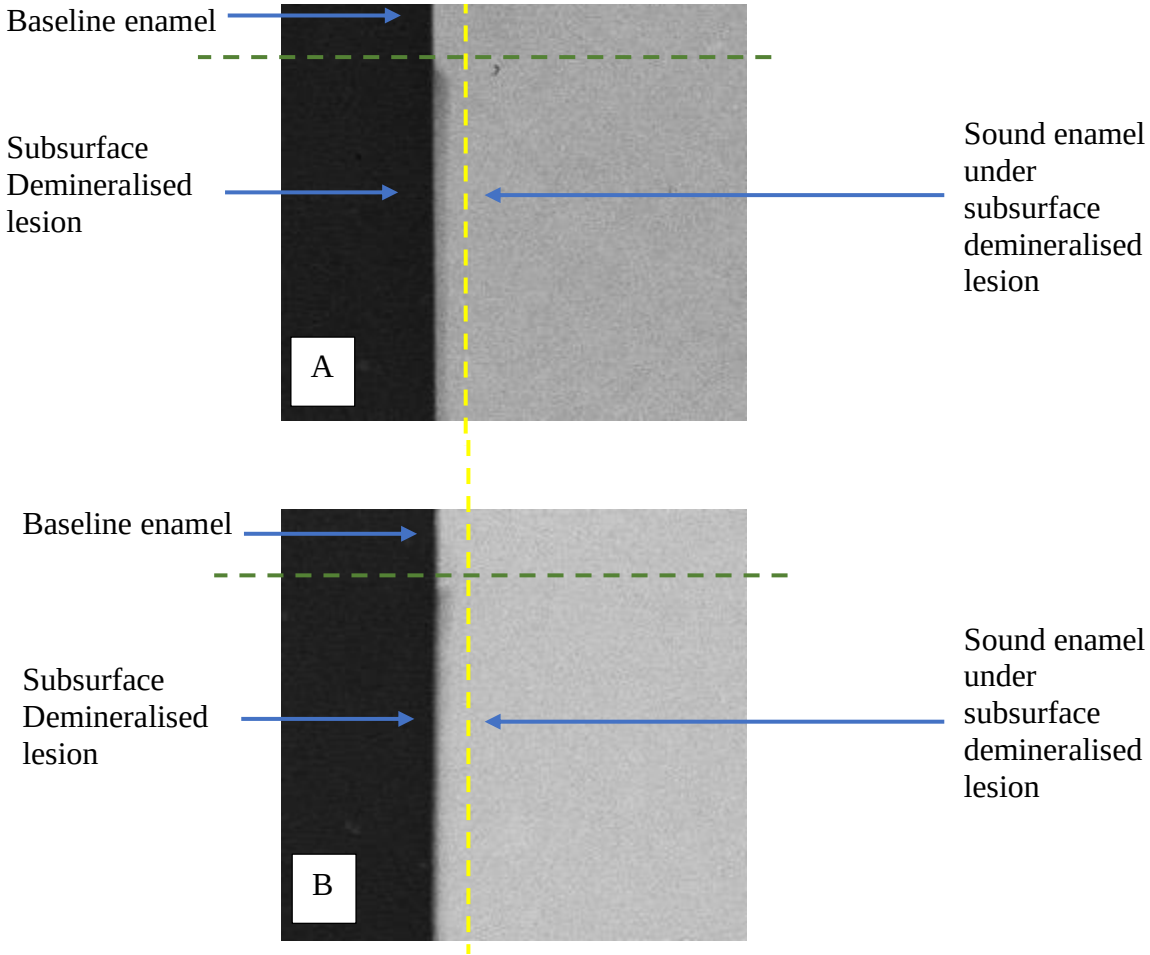


Figure 3.14 TMR images of samples (A,B) in P6 demonstrating subsurface demineralisation

The P6 cycling protocol did not result in the samples coming through the cycle without any enamel demineralisation. A number of key variables, such as time in artificial saliva, time in demineralisation solution and overall duration of each cycle, were adjusted yet the P6 model was not achieving the aims of our study to design a novel model to replicate a 24-hour dietary cycle.

After much discussion and further search of the relevant literature, it was agreed upon by the research team that the main contributing factor is the acidic pH of the demineralising meal solution. A pH 4.5 demineralisation solution was used throughout from P1 to P6 to represent the fall in salivary pH below the critical pH during the consumption of an average meal. The demineralising meal solution which was used to this point has previously been used in *in vitro* cariology research undertaken by the Cariology Research team at the University of Liverpool with the primary use being to create artificial carious lesion and thus not designed to accurately represent the pH drop during the consumption of an average meal.

Following further literature search on salivary pH at mealtimes, the study by Ma (2017) suggests that the salivary pH drops to 5.8 for a few minutes following an acidic meal. Humphrey *et al.* (2001) discussed the resting pH of plaque and the role of saliva as a buffering medium. This study demonstrated that the resting pH of plaque (the pH of plaque 2 to 2.5 hours after the last intake of exogenous carbohydrates) is 6 to 7.3. The salivary pH falls to its lowest level to 6.1 approximately 15 minutes after food consumption. Unless there is additional ingestion of fermentable carbohydrates, the pH of plaque gradually returns to its resting pH of 6 to 7.3 (Humphrey *et al.* 2001).

Therefore, taking the existing literature into account, it was evident that the pH of the demineralising meal solution at pH 4.5 was too acidic to represent the drop in salivary pH during the consumption of an average meal.

3.7.7 Pilot study 7 (P7)

The cycling protocol in the previous pilot study (P6) was conducted on a 24-hour basis with the emphasis of an increase in the overall time of the samples within artificial saliva. Following further literature review, as discussed above, in P7 the key

modification to the cycling protocol was a change in the demineralising meal solution to represent the drop in salivary pH during the consumption of a meal.

The initial demineralisation solution at pH 4.5 was replaced with a modified artificial saliva solution at pH 5.8 to represent a meal solution. A modified artificial saliva solution was more appropriate and accurate in representing salivary pH. As this study did not include plaque or oral flora, an artificial saliva solution was a deemed a suitable medium for representing salivary pH during the consumption of a meal.

The contents of the modified artificial saliva at pH 5.8 to represent a meal solution is shown in Table 3.9 below:

Chemical	**Amount**
Potassium dihydrogen orthophosphate	0.299g
1M Calcium Chloride Aqueous Volumetric Solution grade	2.2ml
Glacial Acetic acid	2.85ml
Deionised distilled water	250ml
Potassium Hydroxide (concentrated)	Adjust pH until 5.8 achieved
Sodium Fluoride solution	0.5ml (500µl)

Table 3.9 Composition of the new demineralising meal solution used in P7

The ingredients for the new demineralising meal solution were added to a 1 Litre volumetric flask and the solution was made up to 1litre using distilled deionised water. The time in the modified meal solution was set to 30 minutes. This was based on the Stephan curve (Stephan 1944) and reflects accurately the time taken for the salivary pH to recover following the consumption of carbohydrate meal. The cycling protocol used in P7 is shown in Table 3.10.

Medium	Duration (minutes)
Start of cycle	
Artificial saliva	480
Wash	1
228ppm F solution	2
Wash	1
Meal Solution	30
Wash	1
Artificial Saliva	145
Wash	1
Challenge/Control	10
Wash	1
Artificial Saliva	145
Wash	1
Meal Solution	30
Wash	1
Artificial Saliva	145
Wash	1
Challenge/Control	10
Wash	1
Artificial Saliva	145
Wash	1
Meal Solution	30
Wash	1
Artificial Saliva	145
Wash	1
Challenge/Control	10
Wash	1
Artificial Saliva	145
Wash	1
228ppm F solution	2
Wash	1
End of cycle	

Table 3.10 P7 cycling protocol

P7 Results

The cycling in P7 was conducted for 7, 10 and 14 cycles respectively. Samples were removed and analysed after the completion of 7, 10 and 14 complete 24-hour cycles. As per previous pilot studies, QLF and TMR techniques were employed to assess for mineral loss. QLF analysis confirmed that there was no mineral loss detected in any of the negative control group samples at either 7, 10 or 14 days of cycling. TMR analysis confirmed that all samples came through the cycling without any mineral loss on the exposed enamel window. This was a major breakthrough in the development

of a novel cycling model. QLF and TMR images of different samples are shown in Figure 3.15 and 3.16 after 7, 10 and 14 complete cycles respectively.

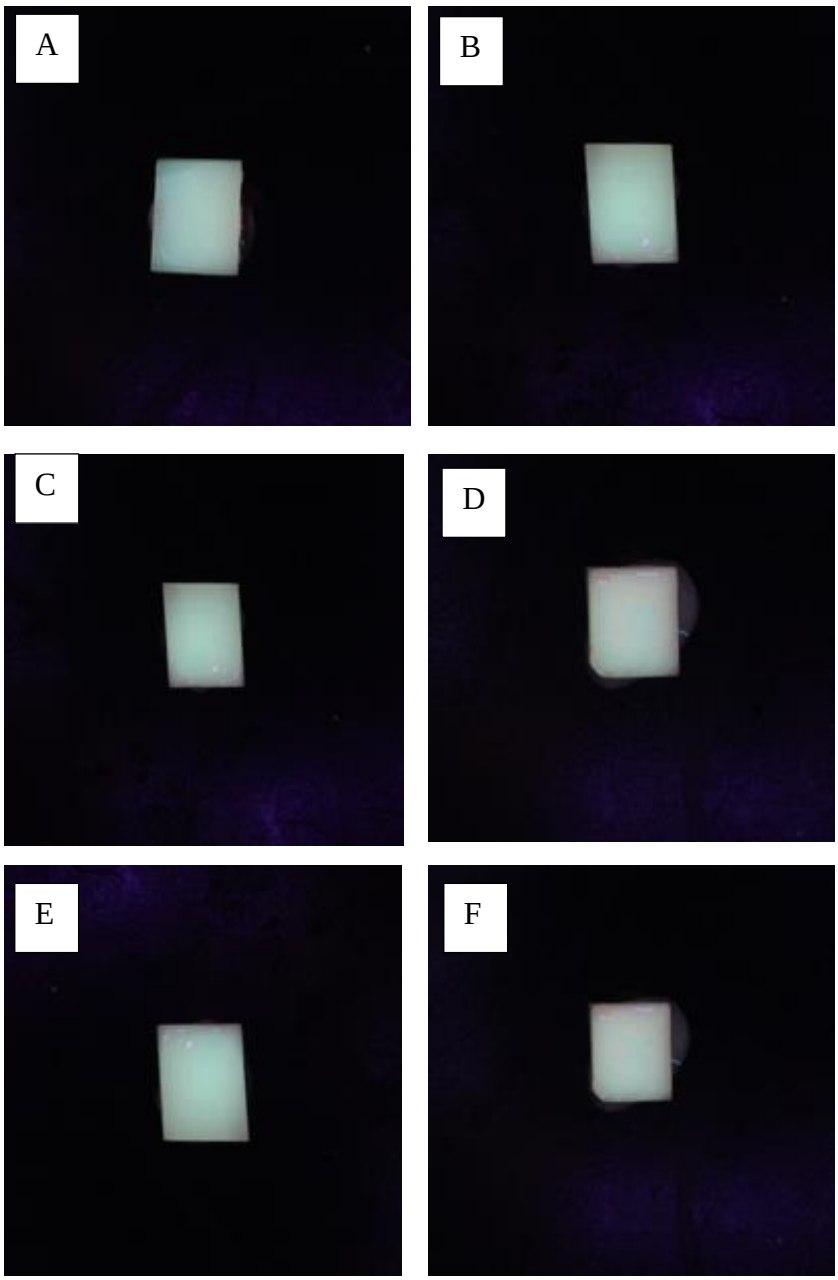


Figure 3.15 QLF images of samples after P7 (A,B- 7 Days, C,D- 10 Days, E,F- 14 Days)

QLF analysis confirmed there was no mineral loss evident on any of the samples for 7, 10 and 14 days of cycling using the P7 cycling protocol.

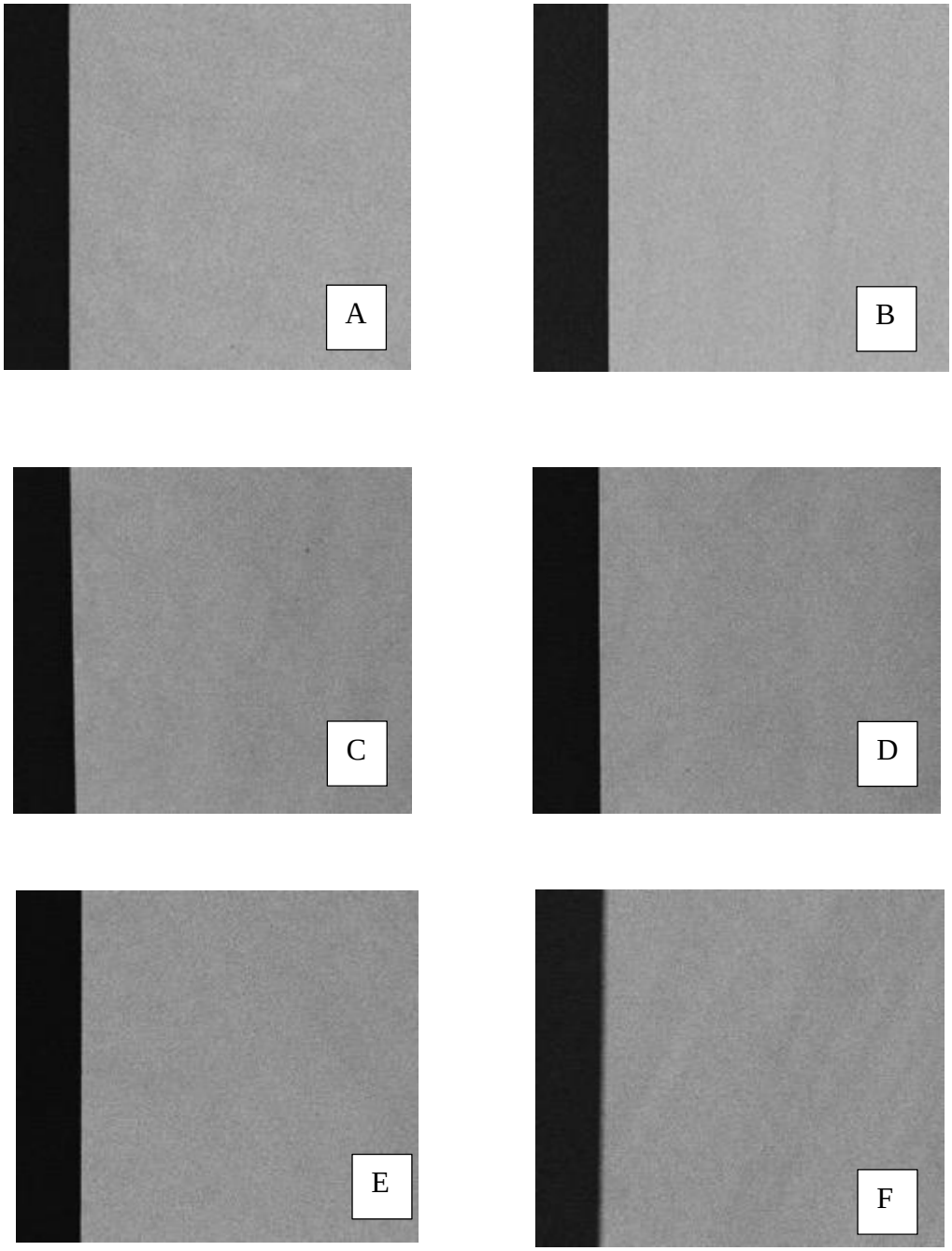


Figure 3.16 TMR images of samples after P7 demonstrating sound surface profiles (A,B - 7 Days, C,D- 10 Days, E,F- 14 Days)

TMR analysis for all samples at 7, 10 and 14 completed cycles confirmed that there was no mineral loss detected. This fulfilled the aims of development of a novel *in vitro* cycling model which represents a 24-hour dietary cycle with respect to pH fluctuations.

3.8 Confirmation of the Oral Health Cycling Model

The results and analysis from P7 confirm that a novel and newly designed *in vitro* cycling model has been developed. The samples in P7 underwent the cycling for 7, 10 and 14 complete 24-hour cycles and did not undergo any demineralisation. Our novel model exposes the dental enamel samples to the various challenges, such as a fluoride solution (to mirror the introduction of fluoride ions during toothbrushing with fluoride toothpaste), a meal solution (to represent the fall in salivary pH during the consumption of a meal), and rest periods within artificial saliva (to represent the buffering capacity of saliva between meals and during non-mealtimes). All of these have been based on the 24-hour cycle reflecting pH fluctuations and challenges within the oral environment. In addition to this, an exposure to a challenge/control medium between meals was established to represent having a drink as a snack.

The results from P7 confirmed that the samples did not have any loss of enamel mineral content within the samples at either 7, 10 and 14 days. Therefore, a decision needed to be made with regards to how many days of cycling will the main study be conducted for. After a review of the literature, it was agreed that the main study will be conducted for 14 complete cycles to represent 14 days. This was based on similar published work (von Fraunhoffer and Rogers 2004, Kitchens and Owens 2007). In addition, conducting the study for 14 days rather than 7 days would give a more longitudinal angle to the results and allow the results to be interpreted for changes over a longer period of time.

To the best of our knowledge, such a novel model has not been described in the literature. The development of the Oral Health cycling model fulfilled the aims of the first phase of this research study. The cycling protocol from P7 was confirmed as the final cycling protocol for the novel model. This novel model has been named as the Oral Health Cycling model.

3.9 Summary

A series of pilot studies were conducted to develop the Oral Health Cycling model. This novel and newly designed pH cycling model represents a 24-hour dietary cycle with respect to pH fluctuations and differing challenges faced by the dentition within the oral cavity.

In the series of pilot studies, the challenge/control medium was focussed on the negative control group samples. The solution used for this was deionised distilled water. The aim was to ensure the cycling model itself did not result in enamel demineralisation. This in turn would allow for other challenges to be incorporated into the cycling protocol to assess their effects on enamel. The next phase of this study was to undertake the main cycling study, incorporating all of the test drinks, positive control, and negative controls within the Oral Health cycling model.

Chapter 4 Results and Analyses

4.0 Introduction

In this section, the main study results and statistical analyses of the study data have been outlined. The initial results report on the chemical analyses of the test drinks. These include pH, Titratable Acidity and Fluoride Analysis of the test drinks. Chemical analysis of the positive control was also carried out. Thereafter, this chapter details the results of the main study in relation to the outcome measure which was mineral loss. ΔF and ΔZ are measured by QLF and TMR respectively. The study results and statistical analyses are presented to assess the sound and demineralised samples separately for the mineral loss. For the sound enamel samples, data was collected after the completion of the main cycling study (T1) and for the demineralised enamel samples, data was collected at Baseline (T0) and at T1.

4.1 Analysis of study drinks

4.1.1 pH

The 3 test drinks selected in this study were Red Bull energy drink, Monster energy drink and Lucozade sport drink. The positive control drink was Coca-Cola (Original), and the negative control was deionised distilled water. The pH of the test drinks and positive control was measured. The pH readings were recorded in triplicate to ensure that an accurate mean could be devised. The mean pH values and standard deviations are described in table 4.1.

Drink	Mean pH	S.D
Red Bull energy	3.41	0.06
Lucozade Sport	3.54	0.02
Monster energy	3.57	0.02
Coca Cola Original	2.53	0.03

Table 4. 1 pH values and standard deviations of the test drinks and positive control

4.1.2 Titratable acidity

Titratable acidity (TA) is a measure of the total acid concentration contained with a food or drink (Sadler *et al.* 2010). TA was performed for the test drinks and the positive control. This was conducted to assess how much volume of 1M sodium hydroxide (NaOH) was required to neutralise 100ml of each test drink and the positive control.

The method for conducting TA measurement has been detailed in Chapter 2. The TA results are shown in table 4.2.

Drink	Mean TA (mL)	Titratable Acidity
Lucozade Sport	7.00	5.25
Monster energy	13.60	10.20
Red Bull energy	13.63	10.22
Coca Cola Original	4.20	3.15

Table 4. 2 Titratable acidity values and standard deviations for the test drinks and positive control

The following formula is used:

TA = (t * m * 75) / v

Where:

m = molarity of NaOH

t = titre of NaOH required (mL0

v = volume of sample used (mL)

Both Red Bull and Monster energy drinks had similar values for TA. TA values for the energy and sports drinks is more than 3 times higher than that of Coca-Cola and this is a significant piece of information. In a previous study by Kitchens and Owen (2007), it was highlighted that a higher TA value is an essential piece of information in understanding why energy drinks have such an impact on tooth structure. The TA measurements is important to understand as the ingredients of energy and sports drinks differs from the positive control Coca-Cola drink used in our study. Modern day energy and sports drinks have multiple organic acids in contrast to Coca-Cola which mainly contains phosphoric and carbonic acid. From the TA analyses, one can presume that the multitude of organic acids within the energy and sports drinks results in a higher TA value.

4.1.3 Fluoride Analysis

Fluoride analysis was conducted to measure the level of fluoride ions within the drinks. The benefits of fluoride within toothpastes have been confirmed by several studies which confirm the effectiveness of fluoridated toothpaste in controlling dental caries

(Carey 2014, Haraszthy *et al.* 2019, Reilly *et al.* 2016, Walsh *et al.* 2019). Although energy and sports drinks are not known for these benefits, it is useful nonetheless to assess the fluoride content as part of the chemical analyses for these drinks. The results for Fluoride content analysis are shown in Table 4.3

Drink	Fluoride value (mv)	µm F^-	ppm F^-
Red Bull energy	190.95	8.33	0.16
Monster energy	237.35	1.43	0.03
Lucozade Sport	212.60	3.65	0.07
Coca Cola Original	196.95	6.62	0.13

Although all ppm values were very low, Red Bull energy drink had the highest ppm F^- whilst Monster had the lowest.

Table 4. 3 Fluoride content analysis of the test drinks and positive control. Fluoride value is in millivolts (mv), Fluoride content is in parts per million (ppm).

4.2 QLF Results

ΔF was measured using the QLF technique to detect mineral loss. The QLF data was divided into Sound samples (QLF-S) and Demineralised samples at baseline (QLF-D). QLF data was collected at baseline (T0) prior to the commencement of the cycling study and following the conclusion of the main study (T1). Statistical analysis of the results was performed using one-way ANOVA for the QLF-S with Post Hoc Tukey Test. To compare the results for the demineralised samples at T0 and T1, ANCOVA was performed and Estimated Means Testing with Sidak adjustment for multiple comparisons for QLF-D data was also conducted. The QLF-S and QLF-D results and statistical analyses are discussed in the relevant sections below.

4.2.1 QLF results for sound baseline enamel samples (QLF-S)

Figure 4.1 to Figure 4.5 show examples of QLF-S samples from each group at end of the main cycling study (T1).

Group 1

Red Bull

Figure 4.1 QLF image of sample in Red Bull group at T1

Group 2

Lucozade Sport

Figure 4.2 QLF image of sample in Lucozade sport at T1

Group 3

Monster

Figure 4.3 QLF image of sample in Monster group at T1

Group 4

Coca-Cola

Figure 4.4 QLF image of sample in Coca-Cola group at T1

Group 5

Deionised water

Figure 4.5 QLF image of sample in Deionised water group at T1

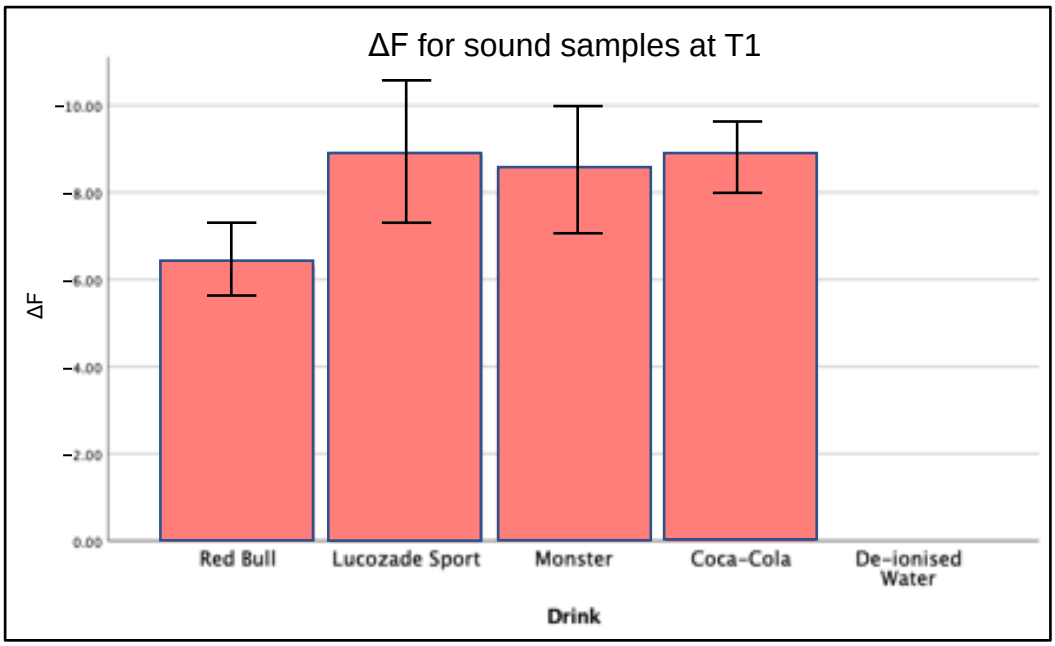
QLF images demonstrated mineral loss ((ΔF) in samples in the Red Bull, Lucozade sport, Monster and Coca-Cola groups respectively. Mineral loss was not detected in any of the samples in the Deionised water group. Table 4.4 shows the QLF (ΔF) data for the sound samples after the completion of the main cycling study (T1). A greater negative number denotes greater mineral loss.

Sample	**Group 1** Red Bull	**Group 2** Lucozade Sport	**Group 3** Monster	**Group 4** Coca-Cola	**Group 5** Deionised Water
1	-6.23	-12.88	-8.09	-9.03	0.00
2	-5.41	-7.99	-7.63	-8.23	0.00
3	-9.52	-9.64	-8.56	-9.26	0.00
4	-6.14	-5.53	-5.71	-7.97	0.00
5	-6.72	-17.32	-12.34	-9.28	0.00

6	-7.77	-8.67	-5.67	-7.27	0.00
7	-7.73	-6.87	-8.59	-9.85	0.00
8	-5.47	-6.81	-9.29	-8.95	0.00
9	-5.32	-7.58	-10.57	-9.28	0.00
10	-7.79	-9.92	-12.81	-12.27	0.00
11	-8.51	-8.89	-12.66	-8.46	0.00
12	-5.52	-8.61	-8.55	-7.09	0.00
13	-9.99	-8.40	-5.12	-10.79	0.00
14	-7.20	-6.64	-7.71	-7.00	0.00
15	-7.53	-7.71	-5.47	-7.76	0.00
Mean	**-7.12**	**-8.90**	**-8.58**	**-8.83**	**0.00**

Table 4.4 Mineral loss (*ΔF)* for QLF-S at T1

The QLF data for each group can be summarised by their respective means. Graph 4.1 demonstrated the mineral loss (ΔF) of the test and control group drinks.



Graph 4.1 *ΔF values for QLF-S at T1*

All of the test drinks as well as the positive control group samples resulted in mineral loss (ΔF) in the sound samples following the cycling study.

4.2.1.1 ANOVA Statistics for QLF-S

To test the null hypothesis that there was no difference in mineral loss amongst the groups, one-way ANOVA was performed for the QLF-S samples. One-way ANOVA statistics are shown below in Table 4.5.

	Sum of Squares	Df	Mean Square	F	P-value
Between Groups	869.954	4	217.489	56.323	<0.001
Within Groups	270.299	70	3.861		
Total	1140.254	74			

Table 4.5 One-way ANOVA for QLF-S

With a P-value of <0.001, one-way ANOVA demonstrated that there was a significant difference for mineral loss (ΔF) overall amongst the 5 groups for QLF-S samples. Therefore, in an effort to ascertain where this difference lies, a Post Hoc Tukey Test was performed.

4.2.1.2 Post Hoc Analysis for QLF-S

Group	Group	Mean Difference	Standard Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
1-Red Bull	2-Lucozade	1.77400	0.71753	0.109	-.2352	3.7832
	3-Monster	1.46133	0.71753	0.260	-.5479	3.4705
	4-Coca-Cola	1.70933	0.71753	0.132	-.2999	3.7185
	5-DH_20	-7.12333*	0.71753	<0.001	-9.1325	-5.1141
2-Lucozade	1-Red Bull	-1.77400	0.71753	0.109	-3.7832	.2352
	3-Monster	-0.31267	0.71753	0.992	-2.3219	1.6965
	4-Coca-Cola	-0.06467	0.71753	1.000	-2.0739	1.9445
	5-DH_20	-8.89733*	0.71753	<0.001	-10.9065	-6.8881

3-Monster	1-Red Bull	-1.46133	0.71753	0.260	-3.4705	.5479
	2-Lucozade	0.31267	0.71753	0.992	-1.6965	2.3219
	4-Coca-Cola	.24800	0.71753	0.997	-1.7612	2.2572
	5-DH_20	-8.58467*	0.71753	<0.001	-10.5939	-6.5755
4-Coca-Cola	1-Red Bull	-1.70933	0.71753	0.132	-3.7185	.2999
	2-Lucozade	0.06467	0.71753	1.000	-1.9445	2.0739
	3-Monster	-0.24800	0.71753	0.997	-2.2572	1.7612
	5-DH_20	-8.83267*	0.71753	<0.001	-10.8419	-6.8235
5-DH_20	1-Red Bull	7.12333*	0.71753	<0.001	5.1141	9.1325
	2-Lucozade	8.89733*	0.71753	<0.001	6.8881	10.9065
	3-Monster	8.58467*	0.71753	<0.001	6.5755	10.5939
	4-Coca-Cola	8.83267*	137.27	<0.001	6.8235	10.8419

Table 4.6 Post Hoc Tukey Test for QLF-S samples

Table 4.6:
Group 5 DH_2O = Deionised water
*Denotes statistically significant difference in mean

The Post Hoc analysis in Table 4.6 demonstrated that there were statistically significant differences for all groups with when compared with group 5 (Deionised water). When the Red Bull, Lucozade Sport, Monster and Coca-Cola samples are compared with samples exposed to deionised water, there was a statistically significant difference for ΔF (p=<0.01). The results and analysis for QLF-S samples confirmed that consumption of the test drinks resulted in mineral loss to a statistically significant level.

When Red Bull, Lucozade Sport and Monster are compared to each other, there were no statistically significant differences detected for ΔF. When considering the mean ΔF values for these test drinks, one can conclude that consumption of all 3 test drinks result in enamel mineral loss as evidenced by the 14 days of cycling in this study.

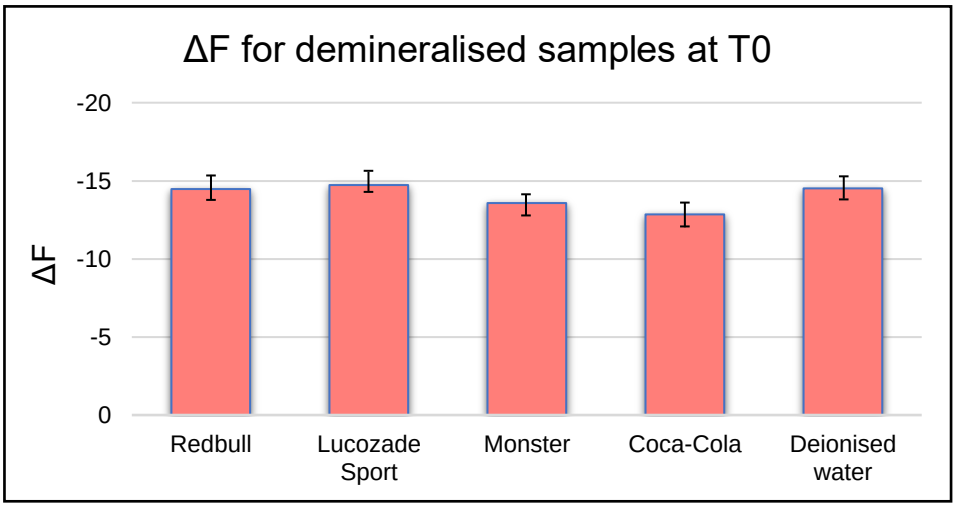
4.2.2 QLF for Demineralised Samples (QLF-D)

As described in previous chapters, this study included both sound enamel samples and enamel samples with subsurface demineralisation. Before the start of the final cycling study, 75 enamel blocks underwent artificial demineralisation to create subsurface caries. This allowed the research team to investigate the effects of the

energy and sports drinks in both sound enamel and in demineralised enamel and thereby increase on the investigative objectives of this in vitro study. Once the demineralised lesions were created (section 2.5.1.8), the samples were randomly allocated to one of 5 groups. Baseline (T0) ΔF data were collected for the demineralised samples (QLF-D). To ensure that there were no differences at baseline, one way Analysis of Variance (ANOVA) was conducted with p = 0.05. The T0 ΔF QLF data for the QLF-D samples is shown in the Table 4.7 and Graph 4.2.

Groups	Count	Sum	Average	Variance
1 Red Bull Energy	15	-216.83	-14.46	24.73
2 Lucozade Sport	15	-220.98	-14.73	5.44
3 Monster Energy	15	-203.82	-13.59	17.98
4 Coca-Cola	15	-192.95	-12.86	16.23
5 Deionised Water	15	-217.68	-14.51	6.74

Table 4.7 Baseline ΔF for demineralised samples for each group at T0



Graph 4.2 *ΔF values for demineralised samples in each group at T0*

Prior to the start of the cycling study, statistical analysis was conducted to determine whether there was any difference in ΔF values amongst the 5 groups at T0. One way Analysis of Variance (ANOVA) to assess this. The summary of the ANOVA statistics (p= >0.05) is shown in Table 4.8.

ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	36.93	4.00	9.23	0.65	0.63	2.50
Within Groups	995.66	70.00	14.22			
Total	1032.59	74.00				

Table 4.8 One-way ANOVA for ΔF data at T0

With the significance set at p = 0.05, ANOVA statistics confirmed that there were no statistical differences in ΔF values for the demineralised samples across the 5 groups (p = 0.63). As the p-value is greater than 0.05, the null hypothesis can be accepted for T0 ΔF data which is to say that there are no differences in ΔF for samples in each of the 5 groups. After randomisation and group allocation of the samples, the main cycling study was conducted. QLF data was collected at the end of the cycling study. Figure 4.6 to Figure 4.10 show examples of QLF-D samples from each group at end of the main cycling study (T1).

Group 1

Red Bull

Figure 4.6 QLF image of sample in Red Bull group at T1

Group 2

Lucozade Sport

Figure 4.7 QLF image of sample in Lucozade sport group at T1

Group 3

Monster

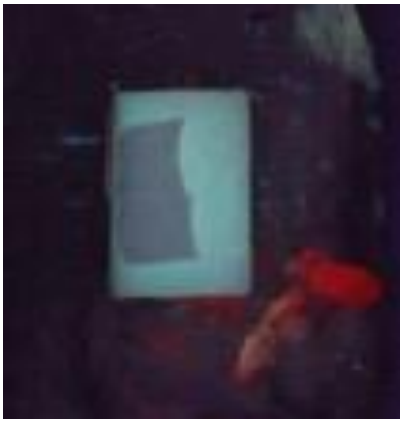


Figure 4.8 QLF image of sample in Monster group at T1

Group 4

Coca-Cola

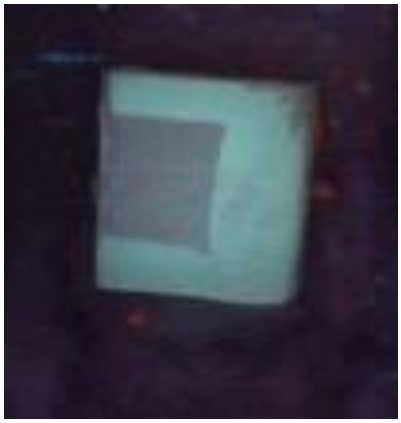


Figure 4.9 QLF image of sample in Coca-Cola group at T1

Group 5

Deionised water

Figure 4.10 QLF image of sample in Deionised water group at T1

The QLF images for all samples confirmed areas of demineralisation. The QLF-D data at T1 was collated and appropriate statistical analyses were conducted.

4.2.2.1 ANOVA Statistics for QLF-D at T1

One-way ANOVA was performed for QLF-D at T1 to detect whether there was a difference between the 5 groups. Table 4.9 shows the ANOVA statistics.

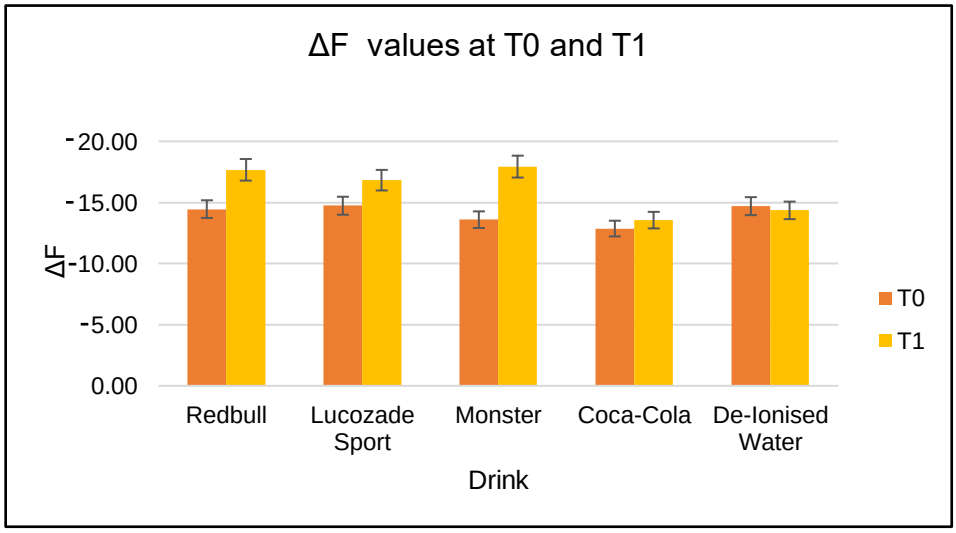
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	238.13	4	59.53	3.08	0.021	2.50
Within Groups	1352.45	70	19.32			
Total	1590.58	74				

Table 4.9 One-way ANOVA for ΔF data at T1

A p-value of 0.021 confirmed that there were statistically significant differences for ΔF at T1 between samples in each group.. Therefore, the null hypothesis can be rejected for T1 QLF-D results.

4.2.2.2 ANCOVA Statistics for QLF-D

The ΔF values for each group at T0 and T1 are shown in the Graph 4.3.



Graph 4.3: *ΔF data for demineralised samples in each group at T0 and T1*

Graph 4.3 demonstrated an increase in ΔF values from T0 to T1 for Red Bull, Lucozade Sport, Monster and Coca-Cola. Samples in the Monster energy group had the largest increase in mineral loss from T0 to T1. There was a marginal decrease in

ΔF value for Deionised water. Using the estimated marginal means for ΔF for each group, ANCOVA was performed to detect where there were statistically significant differences in ΔF between the 5 groups. This analysis is based on the linearly independent pairwise comparisons among the estimated marginal means. This is shown in Table 4.10 below.

Source	Type III Sum of Squares	df	Mean Square	F	P-value
Corrected Model	441.70	5	88.34	5.30	<0.001
Intercept	452.40	1	452.40	27.16	<0.001
ΔF T0	203.58	1	203.58	12.22	<0.001
Drink	216.64	4	54.16	3.25	0.017
Error	1149.24	69	16.6		
Total	20949.99	75			
Corrected Total	1590.94	74			

Table 4.10 ANCOVA for ΔF

A significance level of 0.017 for Drink indicates that there were statistically significant differences in ΔF amongst the 5 groups (Drinks) when independent pairwise comparisons were conducted using the estimated marginal means.

4.2.2.3 Estimated Means Testing Analysis with Sidak adjustment for QLF-D

ANCOVA analysis confirmed that there were statistically significant differences in ΔF between the 5 groups overall at T1 when ΔF at T0 had been adjusted. To further analyse where this difference lies, Post hoc analysis with Sidak adjustment was performed for multiple comparisons using estimated marginal means comparing the 5 groups for QLF-D samples at T1. This analysis is shown in the Table 4.11.

Group	**Group**	**Mean Difference**	**Standard Error**	**P value**	**95% Confidence Interval**	
					Lower Bound	Upper Bound
1-Red Bull	2-Lucozade	-0.973	1.491	0.999	-5.284	3.338

	3-Monster	0.650	1.494	1.000	-3.672	4.971
	4-Coca-Cola	-3.401	1.504	0.239	-7.751	.949
	5-DH_20	-3.428	1.491	0.220	-7.739	.883
2-Lucozade	1-Red Bull	0.973	1.491	0.999	-3.338	5.284
	3-Monster	1.622	1.497	0.964	-2.708	5.953
	4-Coca-Cola	-2.428	1.509	0.696	-6.794	1.937
	5-DH_20	-2.455	1.490	0.666	-6.765	1.854
3-Monster	1-Red Bull	-0.650	1.494	1.000	-4.971	3.672
	2-Lucozade	-1.622	1.497	0.964	-5.953	2.708
	4-Coca-Cola	-4.051	1.493	0.081	-8.369	.268
	5-DH_20	-4.078	1.497	0.079	-8.407	.252
4-Coca-Cola	1-Red Bull	3.401	1.504	0.239	-.949	7.751
	2-Lucozade	2.428	1.509	0.696	-1.937	6.794
	3-Monster	4.051	1.493	0.081	-.268	8.369
	5-DH_20	-0.027	1.509	1.000	-4.390	4.336
5-DH_20	1-Red Bull	3.428	1.491	0.220	-.883	7.739
	2-Lucozade	2.455	1.490	0.666	-1.854	6.765
	3-Monster	4.078	1.497	0.079	-.252	8.407
	4-Coca-Cola	0.027	1.509	1.000	-4.336	4.390

Table 4.11 Post hoc analysis for ΔF data for each group at T1

Table 4.11:
Group 5 DH_2O= Deionised water

When assessing the ΔF changes at T1 for QLF-D samples it was evident that there were no statistically significant results between any of the groups. This was in contrast to the findings for ΔZ (Section 4.3.2.3) where multiple comparison testing demonstrated a statistically significant difference between the test drinks and the negative control. The Sidak adjustment performed on this occasion resulted in non-statistically significant differences for ΔF. Despite this being the case, it is evident by assessing the raw ΔF values that there was an increase in ΔF from T0 to T1. From this, it can be summarised that the consumption of energy and sports drinks leads to further mineral loss when enamel demineralisation is present, although was not to a statistically significant degree.

4.3 TMR Results

The main study data was analysed with an aim to assess the outcome measure, which was mineral loss (ΔZ and ΔF) of the enamel samples. For the sound samples, differences in mineral loss were determined using one-way ANOVA and multiple comparisons between the different groups was determined with Post Hoc Tukey Test. For the demineralised samples, statistical analyses were conducted using ANCOVA and Estimated Means Testing with Sidak adjustment. For the statistical analysis, the significance level was set at $\alpha = 0.05$. All statistical analyses were performed using SPSS version 27 (SPSS Inc, Chicago, Illinois, USA). As per the study methodology, each of the 5 groups had fifteen (n=15) sound samples and fifteen (n=15) samples with demineralised lesions at baseline (T0).

4.3.1 TMR results for sound baseline enamel samples (TMR-S)

Fifteen (15) sound enamel samples were randomly allocated to each of the 5 groups. These enamel samples did not have any form of mineral loss at baseline, therefore any changes in mineral loss were attributed to exposure to the test or control drinks. For the TMR-S samples, five (7%) samples were damaged during the polishing procedure for TMR analysis, three (3) of these were in the Monster group and two (2) in the Coca-Cola group. Despite this, all 5 groups had the minimum number of samples required for data analysis as per the study sample size calculation. Figure 4.11 to Figure 4.15 show examples of TMR-S samples from each group at end of the main cycling study (T1).

Group 1

Red Bull

Figure 4.11 TMR image of sample in Red Bull group at T1

Group 2
Lucozade
Sport

Figure 4.12TMR image of sample in Lucozade sport group at T1

Group 3

Monster

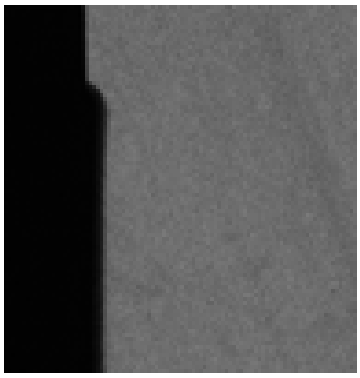


Figure 4.13 TMR image of sample in Monster group at T1

Group 4

Coca-Cola

Figure 4.14 TMR image of sample in Coca-Cola group at T1

Group 5

Deionised

Water

Figure 4.15 TMR image of sample in Deionised water group at T1

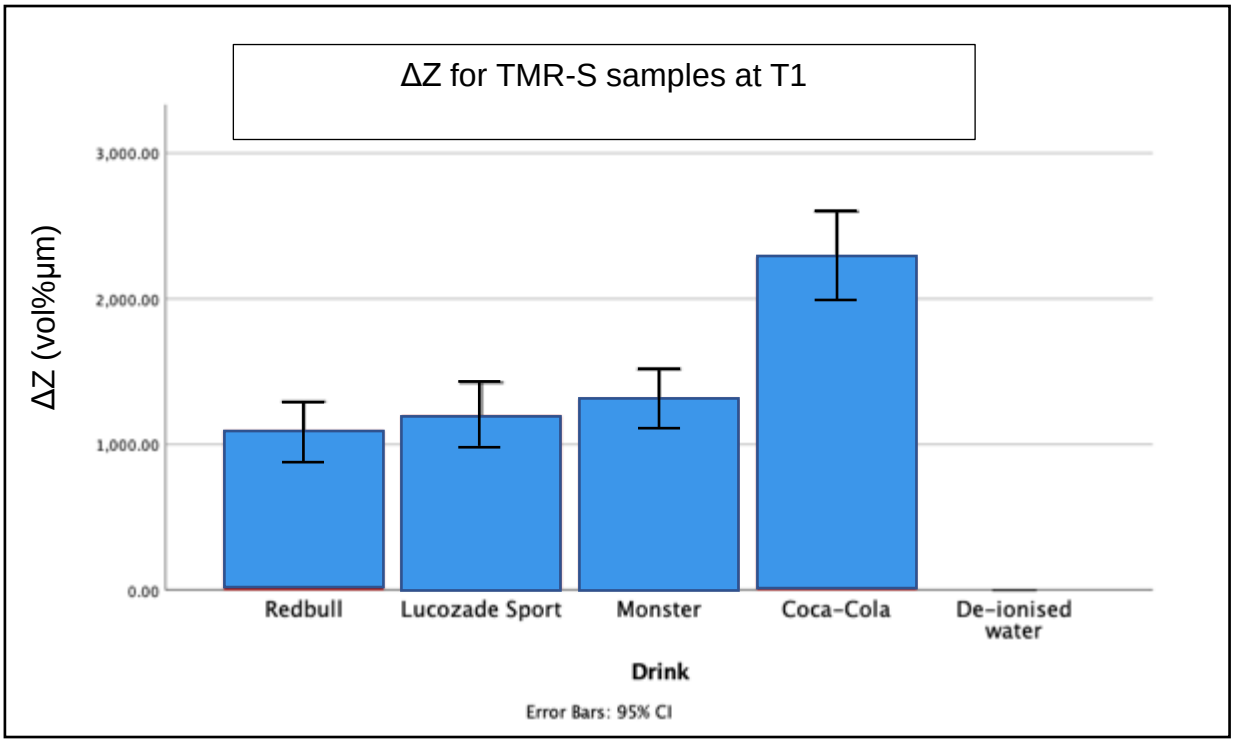
The TMR images show that there is mineral loss evident in Red Bull, Lucozade Sport, Monster and Coca-Cola groups with erosive crater lesions produced in all of these samples. The Deionised water samples did not undergo any surface mineral loss, and this was confirmed with the TMR images and TMR software analysis. Table 4.12 below shows the ΔZ for TMR-S samples by group.

Sample Number	Group 1 Red Bull	Group 2 Lucozade Sport	Group 3 Monster	Group 4 Coca-Cola	Group 5 Deionised Water
1	1470	1035	1230	1840	0
2	1760	1870	1750	2980	0
3	1230	960	1780	2790	0
4	1070	1700	1370	1850	0
5	1080	1150	*	2080	0
6	1520	550	800	2930	0
7	680	1060	*	*	0
8	800	2140	845	2410	0
9	780	910	1415	1460	0
10	1390	1060	960	1810	0
11	570	1195	1450	*	0
12	590	1250	1560	2430	0
13	1320	820	1160	2470	0
14	1080	1050	1420	1790	0
15	1070	1200	*	2900	0
Mean	1094	1197	1312	2288	0

Table 4.12 ΔZ data for TMR-S samples in each group at T1

*Denotes where a sample was lost/damaged during polishing for TMR and ΔZ data could not be obtained.

The raw data has been converted to provide the mean ΔZ for each group. Graph 4.4 illustrated mineral loss (ΔZ) for TMR-S samples in each of the 5 groups



Graph 4.4 *ΔZ data for TMR-S samples for each group at T1*

From the Graph 4.4 above, it is evident that the mean ΔZ for the test drinks are similar, with Monster having the highest ΔZ value of the 3 test drinks. Coca-Cola samples (positive control) had approximately twice the ΔZ compared to the three test drinks, whereas there was no ΔZ detected amongst the deionised water samples (negative control). The group means are interesting but further statistical analyses was required to detect for any significant differences amongst the groups for ΔZ for TMR-S samples.

4.3.1.1 ANOVA Statistics for TMR-S

To calculate whether there is a significant difference between the 5 groups, One-way Analysis of Variance (ANOVA) was calculated using SPSS software. The ANOVA results are shown below in Table 4.13.

	Sum of Squares	Df	Mean Square	F	P-value
Between Groups	37051086.4	4	9262771.59	70.586	<0.01
Within Groups	8529690.77	65	131226.012		
Total	455807771	69			

Table 4.13 ANOVA for TMR-S at T1

ANOVA as demonstrated in Table 4.5 shows that there were significant differences overall between the 5 groups. Based on ANOVA statistics for TMR-S ΔZ, as the P-value is <0.01, we therefore reject the null hypothesis that there are no significant differences between the 5 groups.

4.3.1.2 Post Hoc Analysis for TMR-S

Although ANOVA statistics demonstrated that there are significant differences overall across the 5 groups, it does not show where the significant differences lies. Therefore, for further statistical analysis, a Post Hoc Tukey Test was performed to allow multiple comparisons between the 5 groups for ΔZ for TMR-S samples. The Post Hoc Tukey statistical analysis is shown in Table 4.14.

Group	Group	Mean Difference	Standard Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
1-Red Bull	2 - Lucozade	-102.67	132.28	0.937	-473.81	268.48
	3 - Monster	-217.67	140.30	0.533	-611.32	175.99
	4 - Coca-Cola	-1193.69*	137.27	<0.001	722.86	1465.14
	5 – DH_20	1094.00*	132.28	<0.001	722.86	1465.14
2-Lucozade	1 - Red Bull	102.67	132.28	0.937	-268.48	473.81
	3 - Monster	-115.00	140.30	0.924	-508.66	278.66
	4 - Coca-Cola	-1091.03*	137.27	<0.001	-1476.18	-705.87
	5 - DH_20	1196.67*	132.28	<0.001	825.52	1567.81
3-Monster	1 - Red Bull	217.67	140.30	0.533	-175.99	611.32
	2 - Lucozade	115.00	140.30	0.924	-278.66	508.66
	4 - Coca-Cola	-976.03*	145.02	<0.001	-1382.92	-569.13
	5 - DH_20	1311.67*	140.30	<0.001	918.01	1705.32
4-Coca-Cola	1 - Red Bull	1193.69*	137.27	<0.001	808.54	1578.84
	2 - Lucozade	1091.03*	137.27	<0.001	705.87	1476.18
	3-Monster	976.03*	145.02	<0.001	569.13	1382.92
	5- DH_20	2287.69*	137.27	<0.001	1902.54	2672.84
5-DH_20	1 - Red Bull	-1094.00*	132.28	<0.001	-1465.14	-722.86

	2 – Lucozade	-1196.67*	132.28	<0.001	-1567.81	-825.52
	3 - Monster	-1311.67*	140.30	<0.001	-1705.32	-918.01
	4 - Coca-Cola	-2287.69*	137.27	<0.001	-2672.84	-1902.54

* The mean difference is significant at the 0.05 level.

Table 4.14 Post Hoc Tukey Test for TMR-S *ΔZ* for samples for each group at T1

Table 4.14:
Group 5 DH_2O = Deionised water

The Post Hoc Tukey Test demonstrates that there is significant difference for TMR-S ΔZ values between the Test drink groups (Red Bull, Lucozade Sport and Monster) and the control groups (Coca-Cola and De-ionised Water). However, when Red Bull, Monster energy and Lucozade Sport drinks are compared to each other, there are no significant differences in ΔZ for the TMR-S samples. TMR-S samples for Red Bull, Monster, Lucozade Sport and Coca-Cola (Positive control) all demonstrated significant differences for ΔZ compared to the deionised water group (Negative control).

4.3.2 TMR for Demineralised Samples (TMR-D)

As has been described in previous chapters, this study investigated the effects of the energy and sports drinks on both sound and demineralised enamel. For the demineralised samples, data was collected prior to the start of the study (T0) and following the completion of the main cycling study (T1). Figures 4.16 – 4.20 demonstrate an example of the images captured for TMR-D samples for each group.

Group 1

Red Bull

Figure 4.16 TMR image of sample in Red Bull at T1.

Group 2
Lucozade
Sport

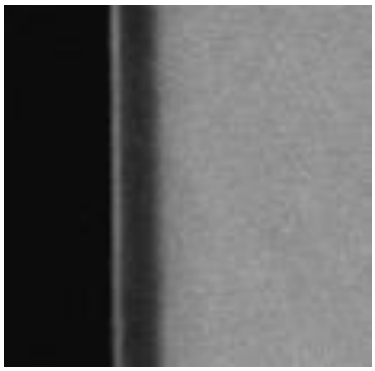


Figure 4.17 TMR image of sample in Lucozade sport group at T1

Group 3
Monster

Figure 4.18 TMR image of sample in Monster group at T1

Group 4
Coca-Cola

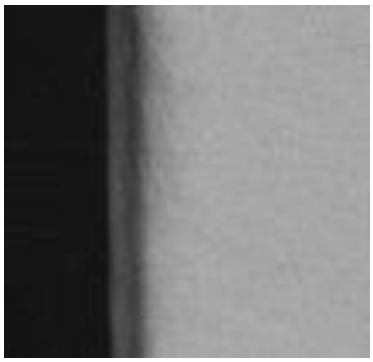


Figure 4.19 TMR image of sample in Coca-Cola group at T1

Group 5
Deionised
Water

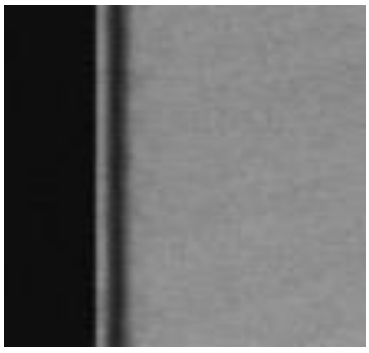


Figure 4.20 TMR image of sample in Deionised water group at T1

After TMR images were captured, TMR analysis was performed to calculate ΔZ values at T1 and adjusting for the values at T0. One-way ANOVA, ANCOVA and Estimated Means Testing with Sidak adjustment were calculated. The TMD-D statistical analyses for ΔZ at T1 are detailed in the sections below.

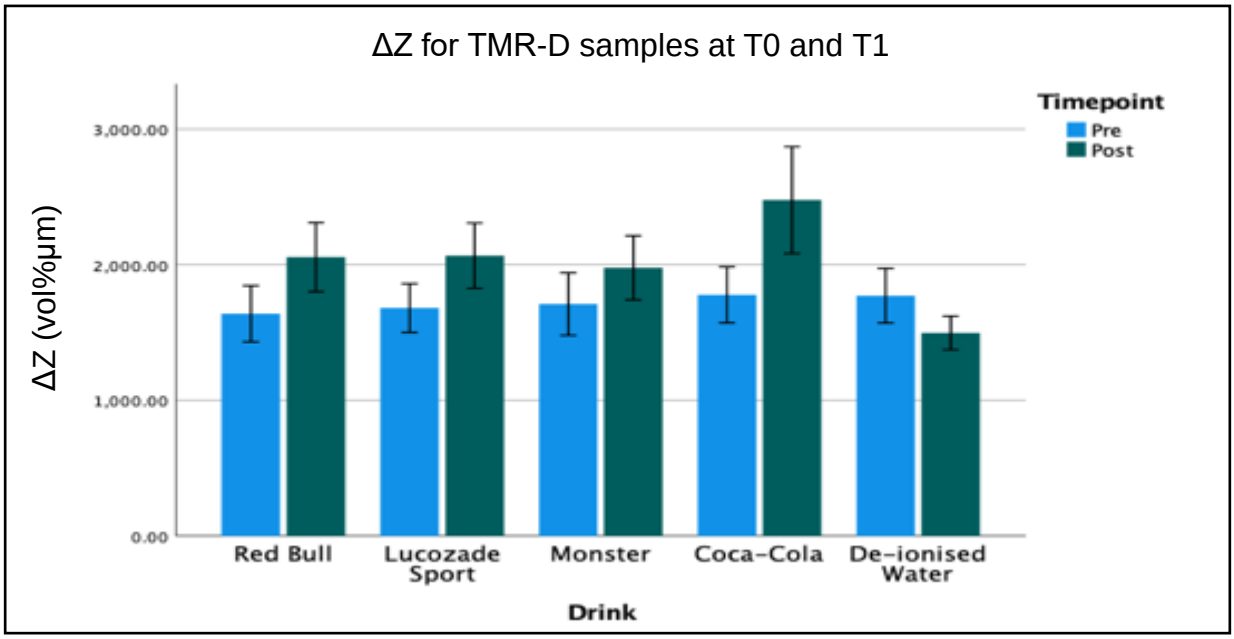
4.3.2.1 ANOVA Statistics for TMR-D

One-way ANOVA was performed to detect for differences in ΔZ amongst the five groups for TMR-D samples. This is shown in Table 4.15.

	Sum of Squares	df	Mean Square	F	P-value
Contrast	6640134.18	4	1660033.55	8.260	<0.001
Error	12661126.8	36	200970.27		

Table 4.15: ANOVA for *ΔZ* TMR-D *samples*

A p-value of <0.001 demonstrates that there. Is a statistically significant difference amongst the 5 groups.



Graph 4.5 Changes in Mean *ΔZ values for Demineralised samples* Pre (T0) and Post (T1) study

Graph 4.5 illustrated the mean group ΔZ changes from T0 to T1. There was an increase in ΔZ for Red Bull, Lucozade Sport, Monster and Coca-Cola. This indicated that consumption of these drinks resulted in further enamel demineralisation if a pre-existing demineralised lesion was present. Amongst the test drinks, the largest increase in mean ΔZ was for Red Bull. Interestingly, there was a decrease in mean

ΔZ for the Deionised water group samples. This suggested that the model developed and designed in this study may have a marginally remineralising effect on enamel with demineralised lesions.

4.3.2.2 ANCOVA Statistics for TMR-D

To compare the differences in ΔZ at T1 Analysis of Covariance (ANCOVA) was performed. ANCOVA is an extension to ANOVA to incorporate a covariate. ANCOVA tests for differences between groups at T1, adjusting for the values at T0. The ANCOVA results uses the estimated marginal means for ΔZ data for pre- (T0) and post-study (T1) values.

Source	Type III Sum of Squares	df	Mean Square	F	P-value
Corrected Model	7380010.5	5	1476002.09	7.34	<0.001
Intercept	64663521.85	1	6463521.85	31.16	<0.001
ΔZ at T0	829137.27	1	829137.27	4.13	0.46
Drink	6640134.18	4	1660033.55	8.26	<0.001
Error	12661126.8	63	200970.27		
Total	302381134	69			
Corrected Total	20041137.2	68			

Table 4.16 ANCOVA for *ΔZ* TMR-D *samples*

The analysis from Table 4.16 demonstrated that ΔZ at T0 had a significance level of 0.46. Therefore, we can conclude that ΔZ at T1 did not have a significant association with ΔZ at T0. In the above ANCOVA analysis from Table 4.16, the dependent variable was ΔZ at T1 and ΔZ at T0 as the covariant. The fixed factor was the Drink (Test drinks and control drinks). From the Table 4.16, it was evident that there were significant differences between ΔZ at T1, adjusting for ΔZ at T0, when comparing the 5 drinks as the significant level is <0.01.

4.3.2.3 Estimated Means Testing Analysis for TMR-D

ANCOVA analysis confirmed that there were statistically significant differences in ΔZ values between the drinks at T1, when ΔZ at T0 have been adjusted. To further

analyse where the difference lies, Post hoc analysis with Sidak adjustment was performed for multiple comparisons using estimated marginal means comparing the 5 groups for TMR-D samples at T1. This analysis is shown in the Table 4.17.

					95% Confidence Interval for Difference	
Group	**Group**	**Mean Difference**	**Standard. Error**	**P-value**	**Lower Bound**	**Upper Bound**
1-Red Bull	2-Lucozade	-4.999	169.466	1.000	-496.634	486.637
	3-Monster	78.171	169.440	1.000	-413.392	569.733
	4-Coca-Cola	-385.453	170.349	0.240	-879.651	108.745
	5-DH_20	597.784*	173.739	<0.001	93.751	1101.816
2-Lucozade	1-Red Bull	4.999	169.466	1.000	-486.637	496.634
	3-Monster	83.169	169.468	1.000	-408.474	574.813
	4-Coca-Cola	-380.455	170.073	0.254	-873.851	112.942
	5-DH_20	602.783*	173.440	<0.001	99.618	1105.947
3-Monster	1-Red Bull	-78.171	169.440	1.000	-569.733	413.392
	2-Lucozade	-83.169	169.468	1.000	-574.813	408.474
	4-Coca-Cola	-463.624	170.366	0.081	-957.871	30.623
	5-DH_20	519.613*	173.757	<0.001	15.528	1023.698
4-Coca-Cola	1-Red Bull	385.453	170.349	0.240	-108.745	879.651
	2-Lucozade	380.455	170.073	0.254	-112.942	873.851
	3-Monster	463.624	170.366	0.081	-30.623	957.871
	5-DH_20	983.237*	172.676	<0.001	482.287	1484.187
5-DH_20	1-Red Bull	-597.784*	173.739	0.010	-1101.816	-93.751
	2-Lucozade	-602.783*	173.440	0.009	-1105.947	-99.618
	3-Monster	-519.613*	173.757	0.039	-1023.698	-15.528
	4-Coca-Cola	-983.237*	172.676	<0.001	-1484.187	-482.287

Table 4.17 Post Hoc Analysis with Sidak Adjustment for multiple comparisons for *ΔZ* TMR-D *samples*

Table 4.17:
Group 5 DH_2O = Deionised water

The results from Table 4.17 demonstrated the analysis of the mean ΔZ values of the 5 groups. As was evident from Table 4.17, there were statistically significant differences for ΔZ between all test drinks and the negative control (p=<0.001). As is evident from Graph 4.5, the data for enamel mineral loss (ΔZ) demonstrated an increase in mineral loss from T0 to T1 ΔZ data amongst Red Bull, Lucozade Sport, Monster and Coca-Cola. We carried out further analysis using multiple comparisons with Sidak adjustment (Table 4.17) and our study confirmed that the consumption energy and sports drinks led to further enamel mineral loss which was statistically significant when compared with the negative control group for samples with baseline demineralisation. This was a clinically significant finding and the results and analyses for TMR-D samples can be utilised to better inform the population regarding the risks of consuming the energy and sport drinks investigated in this study. Interestingly, the post hoc analysis demonstrated that there was no statistically significant difference for mineral loss in TMR-D samples between the test drinks and the positive control (Coca-Cola). Coca-Cola is an acidic drink and numerous studies have been conducted to investigate the harmful effects on teeth (Santos *et al.* 2019, Saads Carvalho and Lussi 2020). The fact that there were no significant differences in mineral loss between Red Bull, Monster, Lucozade Sport and Coca-Cola was an interesting finding and demonstrated the equally harmful effects of the consumption of energy and sports drinks on teeth with pre-existing demineralisation.

This study evaluated whether there was a difference between the energy drinks (Red Bull and Monster) and sports drink (Lucozade Sport). Post Hoc analysis with Sidak adjustment confirmed that there were no statistically significant differences amongst the Energy and Sports drinks for mineral loss for TMR-D samples. Energy and sports drinks differ in regard to their respective ingredients. Sports drinks are marketed as an isotonic drink which help to keep the consumer hydrated (Ostrowska 2016). However, as the results and analyses from this study for TMR-D samples demonstrated, Lucozade Sport was not very different in comparison to the energy drinks in terms of enamel mineral loss and its consumption led to an increase in enamel mineral loss when a pre-existing demineralised lesion is present. There were no statistically significant differences for enamel mineral loss between Red Bull and Monster (p= 1.00).

4.4 Summary of findings

In the sound enamel samples, both QLF and TMR data, and statistical analyses confirmed that the consumption of energy and sports drinks investigated in this study led to an increase in mineral loss (ΔF and ΔZ) to a statistically significant degree when compared with the negative control. Therefore, the null hypothesis that consumption of energy and sports drinks does not lead to enamel mineral loss in sound enamel samples can be rejected. This is a clinically important finding. This led to the conclusion that consumption of Red Bull, Lucozade Sport and Monster led to significant enamel mineral loss for sound enamel samples.

For the demineralised samples, QLF data and subsequent statistical analyses did not detect statistically significant differences in ΔF between the 5 groups T1. This was an interesting finding and was in contrast to the TMR analysis in that there were no differences detected between the test and control drinks. TMR data and statistical analyses confirmed that consumption of energy and sports drinks investigated in this study led to an increase in ΔZ to a statistically significant degree when compared with the negative control. This is a key finding, and this information can be utilised to better inform the public and patients, especially Orthodontic patients undergoing fixed appliance therapy where there is already a risk of enamel demineralisation during the course of their Orthodontic treatment. This key finding can be used in the consent process to better inform patients of the risks of the test drinks.

Chapter 5 Discussion and Interpretations

5.0 Introduction

In this chapter the interpretations of the main study results and statistical analyses of the study data are discussed and outlined with an aim to compare and contrast these results with similarly published research.

Thereafter the main strengths and limitations of this study are summarised. The strengths and limitations of this study are important for internal and external validity. This section will also address the implications for clinical practice and discuss future research that may be conducted based on this current study. The outcome measure in this study was enamel mineral loss (ΔZ and ΔF as measured using the TMR and QLF techniques respectively).

5.1 Main findings and Interpretation

The main study results were divided firstly into the Chemical Analysis of the test drinks that were used in the main study and secondly the enamel mineral loss (ΔZ and ΔF) results from the main cycling study. The main study results are outlined according to the outcome measure. The TMR and QLF data was separated into sound enamel samples and demineralised enamel samples.

Chemical analysis showed that Redbull, Monster and Lucozade Sport were all acidic drinks with low pH values (ranging from 3.41 to 3.57). Redbull and Monster energy drinks had similar Titratable acidity (TA) values (13.63ml and 13.60ml respectively) and were much higher than that of Lucozade Sport (7.00ml) and Coca-Cola (4.20ml). It has been reported that TA and pH remain the most important chemical parameters in determining the erosive potential of acidic drinks (Ablal *et al. 2009*). The higher TA values for the energy drinks was similar to that found by Ehlen *et al. (2008)* who reported much higher TA values for energy drinks compared to regular soda or sports drinks. Chemical Analysis of the test drinks in our study was comparable to that found by Kitchens and Owens (2007*)* where the TA values for Redbull energy drink and Gatorade Sports drink was significantly higher than Coca-Cola. This indicates an increased potential for enamel erosion to occur. The ingredients of the respective drinks play an important role in their ability to cause enamel mineral loss. Kitchens and Owens (2007) summarised that the high degree of enamel dissolution from the energy and sports drinks is primarily caused by the addition of refined carbohydrates (such as

sucrose and glucose) and this in turn promotes acid production. In addition, energy drinks in our study had a combination of different acids within their ingredients, which resulted in a greater volume of base required to neutralise the acids. This subsequently resulted in higher TA values for the energy drinks (Redbull and Monster energy) when compared with the Sports drink (Lucozade Sport) and the positive control used in this study.

When compared with published work assessing the effects of energy and sports drinks on teeth the current study had comparable results and conclusions for both chemical analysis of the test drinks and enamel mineral loss resultant from exposure to the test drinks. The study by Silva *et al. (2021)* assessed the influence of 3 energy drinks on enamel. Similar to our study, they used Coca-Cola as a positive control. The results for chemical analysis of the test drinks were similar to those found in our study. Silva *et al. (2021)* reported Monster energy drink as having the highest TA value amongst their energy drinks used in their study. In our study, when energy drinks are compared, we also found Monster had the lowest titratable acidity value, although it was marginally less than that of Redbull energy drink. All of the test drinks in the current study had TA values higher than Coca-Cola and this was the same in the study by Silva *et al. (2021)*. Moreover Silva *et al. (2021)* concluded that all energy drinks examined were erosive to tooth enamel and we found the same results and can draw the same conclusion from our study.

It has been reported that the number of different acids and type of acids within a drink are important considerations. Meurman *et al.* (1990) found that citric-acid containing drinks had a higher erosive potential than malic acid containing drinks. It was interesting to note that all 3 test drinks had citric acid within their ingredients which may explain the high erosive potential of these drinks as well as the relatively high TA value. Fluoride Analysis confirmed that all test drinks had a very low amount of fluoride with Redbull having the highest at 0.16ppm F^-/100ml and Monster energy drink with the lowest at 0.0316ppm F^-/100ml. The fluoride content for these drinks was insignificant and such low levels of fluoride were not of any benefit.

The current study demonstrated that sound enamel samples that were exposed to the test drinks and positive control resulted in erosive lesions after 14 days of *in vitro* cycling using the Oral Health cycling model. When samples that were in the test drinks' groups were assessed, Monster energy drink group resulted in the highest degree of mineral loss (erosion). Compared to the negative control group, all test drink samples had statistically significant amount of mineral loss in the form of erosion. This was after 14 days of cycling *in vitro*. There were no significant differences detected amongst the 3 test drinks. The results from our study were comparable with the results from von Fraunhofer and Rogers 2004, Coombes 2005 and von Fraunhofer *et al.* 2006 where all energy drinks and sports drinks tested in the respective studies resulted in enamel erosion when exposed to sound enamel samples.

TMR analysis confirmed that all test drinks samples resulted in further demineralisation (ΔZ) in the demineralised enamel samples. When compared with the negative control group, the Redbull, Monster and Lucozade Sport samples all had a statistically significant amount of mineral loss after 14 days of cycling *in vitro*. This demonstrated that the consumption of energy and sports results in further demineralisation and had negative effects on enamel mineralisation where a pre-existing demineralised lesion was present. This was an important finding especially considering that at the time of writing there wasn't any literature published which has investigated the effects of energy and sports drinks in teeth with pre-existing demineralised lesions. The current study confirmed that the consumption of energy and sports drinks led to further demineralisation where a demineralised lesion was present at baseline.

QLF analysis confirmed that all test drinks resulted in mineral loss (ΔF) for the sound enamel samples after 14 days of cycling using the Oral Health cycling model *in vitro*. The degree of mineral loss in the sound enamel samples was statistically significant for all test drinks when compared with the negative control group. There were no statistically significant differences detected amongst Redbull, Monster and Lucozade Sports drinks. However, Lucozade Sport demonstrated the highest mineral loss value in sound enamel samples. This is a very interesting finding and showed the negative effects of sports drinks such as Lucozade Sport.

Furthermore, QLF analysis confirmed that all test group demineralised samples had increased mineral loss when compared to the negative control group. Demineralised samples exposed to Monster energy drink had the highest mineral loss value although there were no statistically significant differences amongst the rest of the test drinks.

The erosive potential of the test drinks used in this study is evident from the results on the sound enamel samples. TMR analysis confirmed that Redbull, Monster and Lucozade Sport all resulted in erosion in the sound enamel samples. When compared with the negative control, the degree of mineral loss (resulting in erosive lesions) was statistically significant. The *in vitro* study by Kitchens and Owens (2007) assessed the effects of carbonated beverages, coffee, sports and high energy drinks, and bottled water on the in vitro erosion characteristics of dental enamel. The study by Kitchens and Owens (2007) included Redbull, Gatorade Sports drink and Coca-Cola. In terms of measuring degree of erosion, they analysed surface roughness by using Profilometer technique. Despite the different technique used, that study concluded that both carbonated and non-carbonated beverages displayed a significant erosive effect on dental enamel. This was a similar finding to our study in that all test drinks resulted in enamel erosion when compared to the negative control group.

It is well established that diet is an important aetiological factor for oral diseases such as dental caries and dental erosion (Moynihan 2005). The current study found that the Redbull, Monster and Lucozade Sport drinks all had the potential to erode sound enamel surfaces and the main study results confirmed that the consumption of the energy and sports drinks resulted in significant erosion over a 14-day cycling period. This is a significant finding and is an important consideration for dental clinicians and nutritionists. The current study assessed Lucozade Sport which is the number one selling sports drink in the U.K. and the main study results have demonstrated that Lucozade Sport has a similar erosive potential to that of the energy drinks.

Sports drinks are thought of as a healthier option and are promoted for use during physical activity to aid optimal physical performance. This may lead to a perception that Sports drinks are not as harmful to the dentition when compared with other carbonated drinks such as Coca-Cola and Energy drinks. Milosevic *et al.* (1997) report

on a descriptive prevalence study of tooth wear and caries experience amongst competitive swimmers and cyclists. They found that the pattern of sports drink consumption was different between the two groups and tooth wear was significantly more frequent amongst cyclists. Although an association between caries/erosive tooth wear and sports drink consumption was not found, the authors did however warn of the erosive potential of sports drinks, and this must be borne in mind as an aetiological factor for erosion in young people (Milosevic *et al. 1997*).

There are a number of published studies which have reported on an association between acidic drinks consumption (especially isotonic sports drinks) and the prevalence of dental erosion (Coombes 2005, Noble *et al. 2011,* Tahmassebi and BaniHani 2020). Sports drinks have a higher mineral content compared to pure mineral water, but these drinks are often supplemented with acidic additives (Attin *et al. 2021*). The addition of acids results in these drinks having a demineralising effect which promotes the development of dental erosion (Shellis *et al. 2004*).

Therefore, it is of concern that there has been significant increase in the sales of sports drinks in recent years (Tahmassebi and BaniHani 2020). Consumers should be informed of these negative risks to their oral health. In addition, consumers of sports drinks should think of them as acidic drinks and not as a healthier option during periods of physical exertion. Tooth wear (as a result of dietary acids) remains a significant oral health problem in the U.K. (O'Toole *et al. 2018*) and coupled with increasing sales of energy and sports drinks, much work has to be done to limit the negative effects of these drinks. This should take shape in the form of more preventative dentistry, with a particular focus on providing targeted dietary advice to patients to raise awareness of the risks of mineral loss associated with the consumption of energy and sports drinks.

Dental caries remains one of the most prevalent diseases both in the U.K and globally (NHS Digital 2011, NHS Digital 2015, Marenes *et al. 2013*) and the aetiology is well known (Pitts *et al. 2017,* Machiulskiene *et al. 2020*). One of the novel factors of this current study was the investigation of the effects of the test drinks on demineralised enamel. By producing artificial carious lesions (subsurface demineralisation) at baseline and then incorporating them into the main study to gauge the effects of the

test drinks on these demineralised enamel samples, this study was able to test an aspect of energy and sports drinks which has not been extensively reported on in the literature. At the time of writing, there was limited published work on the effects of these drinks on teeth with White Spot Lesions (WSLs) (Drozda *2014*). This current study confirmed that the consumption of the test drinks over a 14-day cycling period resulted in further demineralisation amongst the enamel samples with baseline subsurface demineralisation. It was interesting to note that with Post Hoc analysis of the TMR data for the demineralised samples, there was no statistically significant difference for mineral loss between the Redbull, Monster, Lucozade, and Coca-Cola. This confirmed that all of these drinks have a negative effect on teeth with WSLs and consumers should be made aware of the harmful effects of the energy and sports drinks. Diet is a key aetiological factor in caries disease process and therefore appropriate dietary advise should be provided by dental professionals to their patients, especially when in patients with a high risk of WSLs such as Orthodontic patients undergoing fixed appliance therapy.

5.2 Strengths

This study describes a novel cycling model which simulates the pH fluctuations within the oral cavity over a 24-hour period. The Oral Health cycling model takes into account the factors which contribute towards pH fluctuations in the oral environment. The model includes exposure to a fluoride solution to simulate introduction of fluoride ions during toothbrushing with a fluoridated toothpaste, artificial saliva acting as a buffer and a demineralising meal solution to represent a drop in pH during the consumption of a meal. These solutions reflect the various solutions and associated pH changes within the oral cavity over a 24-hour cycle. The consumption of an average meal and the subsequent fall and recovery in pH represents the shifting balance between a net remineralising and demineralising state in the oral cavity and thus the Oral Health cycling model is representative of the pH fluctuations encountered in the oral environment. At the time of writing this work, the current literature does not include such a cycling model, therefore one of the key strengths of this piece of work has been development of this novel and newly designed cycling model.

The literature includes many published studies on the negative effects of the consumption of energy and sports drinks. As such, it would not be ethical to conduct

an *in vivo* or *in situ* study to conduct this investigation on human participants. Due to these ethical barriers, the *in vitro* nature of this study has allowed our research team to investigate and answer clinically relevant questions.

The literature includes several studies investigating the effects of energy and sports drinks on enamel but there is a lack of research undertaken on the effects of these drinks on teeth with early demineralisation/White Spot Lesions (WSLs). Therefore, another strength of this current study is the investigation into how these drinks effect the enamel in teeth with WSLs. If recognised at an early stage, demineralisation can be arrested and therefore it is important to be aware of factors which may accelerate further mineral loss. This study has found a correlation between the consumption of energy and sports drinks and an increase in mineral loss amongst the demineralised enamel samples. This is a clinically important finding and the results from this study will better inform dental clinicians and patients of the risks of consuming these drinks, particularly in patients at an increased risk of dental caries and/or demineralisation (such as Orthodontic patients undergoing fixed appliance therapy). It is anticipated that this study will pave the way for further research into the effects of the test drinks on teeth with WSLs.

Another strength of this study is its generalisability. The results can be applied to the general population or anyone who consumes these drinks. It does not particularly focus on just one cohort of the population and therefore the results are generalisable. In addition, this study investigated the effects of energy and sports drinks on both sound and demineralised enamel. The primary author has conducted specialty training in Orthodontics and one of the main risks with orthodontic treatment with fixed appliances is enamel demineralisation. Although the enamel samples did not have fixed orthodontic brackets bonded on the testing surface, the results of this study are relevant nonetheless for orthodontic patients, as many orthodontic patients who have fixed appliance treatment will end up with enamel demineralisation. Therefore, this study can be used for orthodontic population as it has assessed the risks of consuming energy and sports drinks in teeth with WSLs. The results can be extrapolated to provide appropriate preventative advice to patients undergoing fixed orthodontic appliance therapy.

5.3 Limitations

The main limitation of this study was the *in vitro* cycling model used. The cycling model was highly controlled, allowing the operator to control the pH of the solutions used, frequency and duration of pH challenges and exposure to the various test and control drinks. The cycling protocol exposed the enamel samples to the various solutions in the Oral Health cycling model protocol, but it did not take into account the rate of drink consumption, agitation of the solution within the mouth during drinking, as well as the length of swallow. These are limitations of this study and are linked to the *in vitro* experimental design.

This study was undertaken using bovine teeth and although bovine teeth are the most commonly used substitute for human teeth in dental research (Yassen *et al.* 2011), there are limitations to using bovine teeth in this study. The chemical and morphological structure of bovine and human teeth is different, and one can argue that the effects of the test drinks may be different on human enamel. Davidson *et al.* (1973) reported that the calcium content by weight of bovine tooth enamel was 37.6% and human tooth enamel was 36.8%. Additionally, this study reported that the calcium distribution was more homogenous in bovine enamel compared to human enamel. Featherstone and Mellberg (1981) conducted an *in vitro* study to compare the rates of artificial caries lesions in permanent human and bovine teeth and found that caries lesion progressed twice as fast in bovine enamel compared with human enamel. Despite bovine enamel having many advantages, for the reasons mentioned above, one of the limitations of our study was the use of bovine enamel in place of human enamel.

It would have been interesting to include plaque/oral bacterial microflora to teeth used in this study, particularly as the bacterial microflora play an important role in the aetiology of dental caries. This would have also increased the validity of the study further allowing all cariogenic aetiological factors to be included in the investigations. With that said, there is scope for future research to include bacterial microflora into the study model. Another limitation of this study was that the cycling was conducted with all solutions at room temperature rather than at body temperature which would have been more reflective of the temperature within the oral environment. It may have been more representative to have all the solutions at normal oral cavity temperature during

the *in vitro* cycling to more accurately mirror the temperature within the oral environment and increase the validity of the study results.

5.4 Implications for clinical practice

The results from this study outlined that consumption of the test drinks on a regular basis results in erosion in sound teeth and further demineralisation in teeth with WSLs compared to the negative control drink. This has important clinical implications for dental clinicians and the practice of dentistry. With significantly increasing sales of energy and sports drinks, there may be a further increase in the prevalence dental erosion and caries within the general population. Dental clinicians should be aware of the negative effects of energy and sports drinks and should consider their patients' diet as an important aetiological factor in dental disease such as erosion and caries. A strategy should be devised on taking appropriate diet history and providing appropriate dietary advice so patients can limit the consumption of the test drinks. Dental clinicians should consider their patients' diets more carefully and adopt a preventative approach by providing regular and focussed dietary advice to patients. Orthodontic patients undergoing fixed appliance treatment are particularly at risk of enamel demineralisation. This may be further compounded by consumption of energy and sports drinks. Therefore, Orthodontic clinicians should provide frequent and targeted dietary advice to their patients to limit their risks of enamel demineralisation.

With increasing sales of energy and sports drinks in the U.K. over the past few years, it is imperative that Dental and Public Health bodies strategise on how to limit the negative effects of these drinks on the oral cavity. This should include working in partnership with the U.K. Government Department of Health and Social Care to introduce measures to raise awareness of the negative effects of these drinks on the dentition. Manufacturers of these drinks market their products with trendy and attractive advertisements but the message of the harmful effects of these drinks is often not highlighted. Therefore, it falls within the remit of public health bodies to raise the awareness of the oral health risks.

5.5 Future Research

The Oral Health cycling model has great potential for future cariology research with a particular focus on remineralisation of early carious lesions and fluoride reactivity. The

current study assessed the effects of energy and sports drinks on enamel and the results confirmed the negative effects of these drinks. Future research should be conducted to gauge whether these negative effects can be prevented by incorporating additional remineralising agents into the cycling model. There is scope to investigate more beverages, particularly those that are marketed as “diet” or “sugar-free” to assess whether the effects are any different to the original drinks tested in this study.

Furthermore, there is potential to use the Oral Health cycling model to investigate the effects of different levels of calcium concentrations in Fluoride research. Future models can maintain baseline demineralised enamel samples to assess the efficacy of differing remineralising agents/solutions. Further research should also concentrate on further developing and refining the novel cycling model. This should include the use of human enamel, additional of bacterial microflora and accurate temperature control. In addition to this, future research should consider other factors such as salivary flow rate, salivary content, viscosity of the solutions used and incorporate this into a refined cycling model. Previous research that the type of acids within drinks can have an effect on their erosive potential (Kitchens and Owens 2007). Future research into the effects of energy and sports drinks can dive into the ingredients and focus on the effects of specific acids with an aim to identify the acid with the most and/or least damaging effect on teeth. The manufacturers of the test drinks can fund such research in an effort to restrict the harmful effects of their products by identifying specific acids which are less harmful and try to incorporate those acids primarily within their product.

5.6 Conclusions

The current study was a two-phase research project to firstly design a novel *in vitro* cycling model which is representative of the challenges faced within the oral environment over a 24-hour period and secondly to use the model to investigate the effects of Redbull, Monster and Lucozade Sport on sound and demineralised bovine enamel samples. The findings from this *in vitro* study indicated key factors which were important in the development of the novel cycling model. These factors included the use of a demineralising meal solution at pH 5.8 which was representative of salivary pH during the consumption of an average meal. Artificial saliva was an important addition to the model as it was a replacement for natural saliva to act as a buffer. The length and duration of the cycling protocol were also important factors, as the series of

pilot studies showed that shorter cycles resulted in demineralisation of samples exposed to a negative control group. Over a series of pilot studies and the subsequent alterations made to the cycling protocol such as the factors mentioned above, the current study developed a model which takes into account the various pH fluctuations encountered within the oral environment and does not have a net demineralising effect on samples exposed to a negative control group. The development of this *in vitro* cycling model can be considered to be an innovative method of assessing the effects of the test drinks on sound and demineralised enamel samples. This new method facilitated further *in vitro* research to investigate Redbull energy drink, Monster energy drink and Lucozade Sports drink for their effects on bovine enamel. Using the oral health model and within the limitations of this *in vitro* study, the following conclusions can be drawn relating to the test drinks:

1. Exposure to Red Bull, Monster and Lucozade Sport results in significant enamel mineral loss in sound bovine teeth *in vitro*.
2. Exposure to Red Bull, Monster and Lucozade Sport results in significant enamel mineral loss in demineralised bovine teeth *in vitro*.
3. There is no statistically significant difference between Red Bull and Monster energy drinks when compared to Lucozade Sports drink in terms of enamel mineral loss after 14 days of *in vitro* cycling.

Based on these conclusions, it can be proposed that dental clinicians and other healthcare professionals should be aware of the harmful effects of Red Bull, Monster and Lucozade Sport and adopt an appropriate preventive approach to their clinical practice to prevent further dental disease in patients who consume these drinks.

6.0 Reference list

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