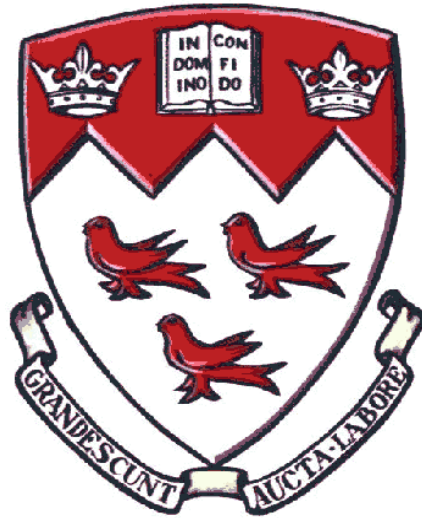


Pop-Colas and Dental Corrosion



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Abbreviations

ICP-OES	Inductively Coupled Plasma with Optical Emission Spectroscopy
IC	Ion Chromatography
SEM	Scanning Electron Microscopy
ESEM	Environmental Scanning Electron Microscopy
VP-SEM	Variable Pressure Scanning Electron Microscopy
AT	Automatic Titrator
pH	Acidity
M	Molar Concentration (Molarity)
ppm	parts per million
ml	Millilitre
Ca	Calcium
P	Phosphate
F	Fluoride
NaOH	Sodium Hydroxide
H₃PO₄	Phosphoric acid
Ca₁₀(PO₄)₆ (OH)₂	Calcium Hydroxyapatite
ECJ	Enamel-Cementum Junction
DIW	De-Ionized Water
GFN-P	Gassy/Flat/Neutralized Pop-Cola
NP-GHA	Non-Porous Granular Hydroxyapatite
MAM-ABS	Man-Made Alcoholic Beverages
MAM-NABS	Man-Made Non-Alcoholic Beverages
DD	Dental Damages
EDTA	Ethylenediaminetetraacetic Acid

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Preface

Dental erosion is a natural occurrence over time; causal factors accelerating this process are important to the dental profession, supporting industries and community at large. This thesis focuses on pop-beverages, especially *acidic pop-colas*, as active promoters in *dental damages*. This research is timely and important, as consumption of pop-cola beverages has increased dramatically during the last two decades (1990-2010). This situation leads not only to increased intake of sugar and obesity resulting in progressive rates of diabetes and cardiovascular diseases in North Americans, but also erosive tooth wear: as Adrian Lussi (2006) notes, “ Erosion is becoming increasingly significant in the long term health of the dentition and the overall well-being of those who suffer its effects” (1).

In the chapters following, a brief history of cola beverages and related new research on pop-colas and teeth, [types of dental damages, selected laboratory-based research comprising both *in-vitro*, *in-vivo* electron microscopy and clinical implications of the effects from exposure to acidic pop-colas], will be presented and discussed.

Chapter 01

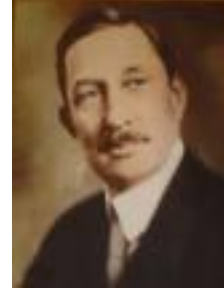
A Short History of Cola Beverages Development



(i) Joseph Priestley
(1733-1804)



(ii) Stith Pemberton
(1831-1888)



(iii) Caleb Bradham
(1867-1934)

Figure 1-1: Major historical people in discoveries of carbonated pop-cola.

(i) Priestly dissolved gas in water. (ii) Pemberton formulated the original Coca-Cola. (iii) Bradham successfully created Pepsi-Cola. See text for further details.

1.1) Introduction

Throughout history, mankind has sought to enhance pleasurable experiences of diet through consumption of nutritional foods and drink. Ancient cultures, from Babylonians to Egyptians, developed juice-making processes from fresh fruits and fermented mead, wine and beer. Natural water played a most important role in daily life for survival, sustenance, and is essential for human development and progress. Natural spring flows were regarded as not only necessary for survival but also to have had health and healing properties. This was a cultural characteristic of the ancient Greeks (~550 BC). Also water as a solvent was used to dissolve discovered substances to which magical curative (medicinal) properties were attributed.

1.2) Soda drinks (Schweppes)

Water-based potions were popular for centuries before the arrival of discoveries that led to creation of pop-drinks. The history of soda-pop or what is also termed as “pop-cola” can be traced back to an English clergyman *Joseph Priestley* [Figure 1-1(i)], who also was a scientist. He conducted experiments in methods of impregnating water with fixed air. His experiments led him to utilize a pump to saturate water with higher amounts of fixed air by compressing gas, and finishing the process of absorption through agitation under pressure. Priestley’s findings were published from his treatise “Directions for impregnating water with fixed air” in 1772. After 10 years of experiments, the first commercially available soda drinks (potable drinks of water with CO₂ dissolved in them) were manufactured by Jacob Scheppe, a jeweler in Geneva. Scheppe was aware of Priestley’s findings, and established a mineral water company in 1790. His business flourished and he moved to London where he opened a successful drinks factory in 1792 (2). The rapid popularity and sales of Schweppes benefited from the medical profession who not only recognized soda water as a substitute for natural spa-waters, but also a reliable supplier of health drinks by his company for patients and the public, as refreshments at the Great Exhibition of London in 1851 (3). The notion that medicinal value was inherent in a good drink was reinforced by adding quinine to soda water. Quinine was assumed to make one’s blood bitter, and consequently not attractive to mosquitoes. Schweppes gained international exposure by distribution of products throughout the British Empire, notably with its quinine-tonic water being served with gin [from which the notorious gin-and-tonic drink derives to this day]. Scheppe’s

success proceeded unchallenged throughout British Colonies during the latter half of the nineteenth century (~1880), but parallel developments were happening “across the pond” in the United States of America. [See below Coca-Cola]. In 1953, Schweppes entered into a partnership with Pepsi-Cola where Pepsi would bottle and sell Schweppes in the USA and Canada, allowing a readily existing distribution network to promote Schweppes, and conversely Schweppes would bottle and sell Pepsi in the UK through its existing system of marketing (4).

1.3) Coca Cola

In the late 1800's [1887-1890] an Atlanta-Georgia USA pharmacist, *John Stith Pemberton* **[Figure1-1(ii)]**, founded Coca-Cola in 1887, selling a kind of concentrated syrup diluted with pop-soda water, at Jacob's Pharmacy as a health tonic for headaches and other ailments. This concoction was derived from a secret formula of spices and herbs including the coca leaf. He recognized it appealed enormously to people as a soft drink, and patents were granted for this pop-cola drink. The Coca-Cola Company was sold in 1891, and rapidly expanded through large-scale distribution after 1900. Early in the Twentieth Century Coca-Cola chemists managed to remove all traces of cocaine from the drink but retained the full symphony of flavors of the original drink. Coca-Cola's strength was its' patent registration, its' name, formula, bottle shape, insignias and brilliant marketing. Its aggressive campaign was successful in claiming Coca-Cola's secret formula could not be imitated by competitors, who spuriously offered poor imitations of the logo, drink and bottle shape (5). Pepsi-Cola was the exception based on its own formula and strategies and was established ~1920.[see below Pepsi-Cola]

In the 1920's the Coca-Cola Bottling Company cast its business activities into the international arena. The Coca-Cola Company created a 'Foreign Department' to establish bottling plants with Coca-Cola know-how and patent controls, embracing local partners in foreign countries to further the companies' business interests.(5). Also the company achieved success through advertising which idolized their iconic bottle shape in the first half of the 20th century. The D'Arcy Advertising Agency, were instructed by Coca Cola to use a philosophy of "Keep it Wholesome" and use straightforward themes with appeal to growing middle classes (6). Coca-Cola gained great visibility to consumers by using brand-identification on cola dispensers situated in towns and cities across the USA. Early vending machines consisted of tubs painted red, emblazoned with the Coca-Cola logo and were filled with ice (7). The metal Vendo-Top cooler followed in the 1930's in which a coin was deposited, a lever pulled and out dropped an ice-cold bottle of pop-cola for the consumer (8). After various upgrades, many coin deposit machines were placed the world over. In the 1980's the company started using the now familiar machines we see today with lighted full panel fronts (9). Coca-Cola's world-wide growth after World War II (1939-1945) was determined by persuasive advertising that tied lifestyle to its brand. To enhance its image as an entrepreneurial socially responsible company, Coca-Cola expanded outside the limits of a beverage industry, to set its role as the most visible soft-drink in the world. Coca-Cola purchased Columbia Pictures Industries in 1982, and introduced its line of Coca-Cola clothing cleverly recruiting individuals to feel a sense of identity with a successful American iconic symbol, and become personal unpaid walking advertisements for the Coca-Cola brand-name (10).

1.4) Pepsi Cola

Coca-Cola became known by the abbreviated name Coke, without any dubious association to the drug cocaine. *Pepsi-Cola* was started as a brand name in New Bern, North Carolina USA by a Pharmacist named *Caleb Bradham* [Figure1-1(iii)](11). His drink “contained pepsin, a digestive enzyme, and was marketed mainly as a stomach soother”(12). “Pepsin” was the etymological origin of the neophyte cola name “Pepsi-Cola”. The company had some initial success but at first went bankrupt, and its trademark was acquired by a *Charles Guth* (1876-1948) who ran an expanding confectionary business in Baltimore and New York State. Having purchased a large candy company in the 1920’s, Guth requested wholesale discount prices from Coca-Cola on 30000 gallons of Coke he was buying from Coca-Cola annually at that time. Coca-Cola refused; consequently Guth ordered his stores to stop selling Coke, revived the Pepsi-Cola name and hired his own chemist to create syrup to taste similar to Coca-Cola. Coca-Cola sued Pepsi and launched a court case in 1933 which Coca-Cola lost, and subsequently Pepsi-Cola began competing directly with Coca-Cola selling in grocery stores countrywide in the USA, and later globally (12). The key to Pepsi expanding its growth in the 1930’s against Coke was their marketing plan to offer double-drink volumes, larger bottle size (12 ounces of Pepsi-Cola versus 6.5 ounces Coca-Cola) but selling at the same price (12). Young people, under the age of thirty, [pre-teens, teens and young adults of both genders] found drinking pop-Colas as a regular beverage, to be most appealing and satisfying. Vigorous and vibrant marketing started in the 1980’s when Pepsi launched taste-tests, showing in advertisements and billing itself as the “new generation” drink.

This appealed to a much younger set of buyers. In the past two decades (1990-2010) both rival companies link themselves through advertising to a variety of sports activities, action events [like hot-air ballooning, sky-diving], and entertainment industries in a battle of advertising wits to sustain their image, markets and brand names. Both companies try to hold onto and expand sales incrementally against each other. Chronic frequency of imbibing, method of drinking, timing of consumption or type and quantity of acid pop-beverages are known to damage teeth (42). The formulae and contents of pop-Cola beverages are now closely guarded industrial secrets, and sparse data about their contents and/or acidity is provided on their consumer packages.

Appreciation of the historical background of the discoveries of cola drinks is important because many different brands are currently (2011) available, and cola drinks constitute a large portion of the beverage industry.

Chapter 02

Dental Damages

2.1) Introduction

Dental damages include attrition, abrasion and erosion, all of which affect the appearance and function of a good dentition. Recent medical science observes oral health as an important factor, not only in keeping and maintaining healthy teeth, but also for playing an important role in overall body health. Healthy teeth, in healthy gums reflect good health. Accordingly, tooth erosion has become a factor of critical importance for dental science, the dental profession and patient well-being. Individual chemical, social, environmental, and behaviour factors may all contribute individually or agonistically to producing dental damages. In this chapter, the aetiology, types of dental damages and implications relating to oral biological research are defined, appraised and discussed.

2.2) Attrition

Definition: - **Attrition**, though contributing to erosion, is itself a specific process occurring over time by “removal of dental hard tissue by tooth-to-tooth contact, without the intervention of foreign substances” (13;14). This is an occurrence witnessed naturally as part of chewing which is a conscious neuromuscular activity, but also from para-functional reflexes such as bruxism (the grinding of teeth) or clenching of teeth (15). However, attrition is understood to be accelerated by other interactions. As Addy et al (2006) discussed, enamel/enamel

(occlusal) attrition under loads is affected by the presence and types of lubricant, noting it is possible water or saline may hold enamel particles within the mouth and contribute to abrasion, termed three body abrasion (13). Measuring tooth wear has been assessed through a number of differing methodologies. First to classify wear by degrees was Broca's 1879 study, which have been used since by researchers. Recent studies take advantage of more developed technology and employ three dimensional and digital imaging to quantify wear on molar teeth (15). The difficulty with long term studies is problematic because teeth continue to erupt through adulthood along with formation of secondary dentine; these natural processes counter attrition or erosion rates and confound measures of volume (15).

2.3) Abrasion

Definition: - **Abrasion** is “ The physical pathological loss of hard tissue from an extraneous force, without bacteria, from anything introduced into the mouth” (15)). Erosion may include tooth abrasion, the word abrasion deriving from the Latin term *abrasum* meaning to ‘scrape off’. The most common and obvious causal factor of dentine abrasion is faulty tooth brushing, [tooth-brush abrasion] particularly a combination of a hard toothbrush and some rough-particled pastes in producing abrasion (13). Abrasion is moderated mainly by tooth-brushes, from strength of brushing-force, length of time-brushed, bristle-softness and stroke-frequency. Studies on abrasion from tooth brushing show widely varying and conflicting results with confounding factors like age, techniques used for brushing (horizontal vs. vertical), and right-handed versus left-handed brushing, all influencing researches

which yield variable results (16;17). After the hardness of the brush used, the second main agent detailed in dentine wear is toothpaste. The role of “relative dental abrasivity” determined by *International Standards Organization* and its effect on dentine wear *in vitro* is shown to be present, while *in situ* studies indicate significant differences in measurements of dentine wear (13;18;19). Also, abrasion may occur from foreign objects placed in the mouth such as dental floss, toothpicks or metal/steel utensils used during eating or as a ‘habit’. Early research used rough pastes and hard bristle brushes *in vitro*, but more current studies demand use of least abrasive paste *in situ*, and stress use of tooth brushes with controlled hardness of bristles (15).

2.4) Erosion

Definition: - **Erosion** in teeth is a “the chemical dissolution of tooth material without bacteria and no cavitation” (42). More specifically, it is in effect the loss of tooth structure by acid dissolution without bacteria (20). According to recent studies, dental erosion is among the most common chronic diseases with high prevalence in children (21). The calcified portions of dental enamel and dentine, though similar chemically (both are constructs of calcium hydroxyapatite crystal), are two very different structures. Both consist of mineral, protein, lipid and water in different ratios, but both are susceptible to effects from the elements in the oral environment (22). *In situ*, saliva plays a critical role; saliva is a saturated solution of phosphates with some calcium ions; saliva at neutral pH is in a supersaturated state in relation to enamel hydroxyapatite. As soon as the pH drops the solubility of Ca^{+} increases. Accordingly, the critical pH for solubility of hydroxyapatite is pH dependent in this

phosphate solution. Lowering pH levels in saliva will cross the saturation point, defined as *the critical pH*, which allows calcium dissolution, and is placed at between pH6.5 and pH5.5. Dissolution may start at 6.5 [depending on the surrounding ions], but below pH5.5 calcium dissolution is definite. Accordingly pH5.5 is considered the critical point for calcium dissolution. Solutions of a lower pH usually have noticeable erosive effects. The inorganic dental matrix is the substance most vulnerable to acid attack. Once surface hard tissue is decalcified, the formed soft layer is more vulnerable to abrasion, and when removed, a repeat cycle of erosion takes effect (42). This situation can also advance to underlying dentin resulting in dissolution at the junction of the peri-tubular and inter-tubular dentine, followed by widening of tubule- luminae (20;23). Please see **Table 2.1** for working concepts of Frangible Terms.

Table 2.1 Glossary of Terms
After Touyz and Mehio 2006 (42)
“Frangibles: A generic collection term describing common damage to hard and soft dento-alveolar tissues at, or adjacent to cervical and peri-cervical tissues; it embraces recession, attrition, abrasion, erosion and demastication affecting hard tissues and also peri-cervical soft gingival tissues.” “Attrition: Loss of hard tooth material from contact with other tooth material.” “Abrasion: The physical loss of hard tissue from an extraneous force, without bacteria, from anything introduced into mouth: e.g. toothbrush abrasion.” “Erosion: Chemical dissolution of tooth material without bacteria and no cavitation.”

In the next two chapters [**Ch.3 & Ch.4**], effects from external dietary sources, specifically of acid pop-cola drinks, and behavioural habits when imbibing these drinks, will be discussed.

Chapter 03

Enamel and Hydroxyapatite Erosion

by de-ionised water, gassed, degassed and neutralized pop-cola.

In vitro and electron-microscopic evidence

Outline

Abstract

Résumé

Introduction

Aim

Methodology

-pH AND BUFFERING.

AUTOMATIC TITRATOR [AT]

RESULTS AND STATISTICS

*-IMMERSION OF TEETH AND NP-GHA IN WATER, AND
FRESH/FLAT/NEUTRAL PEPSI.*

RESULTS AND STATISTICS

-SCANNING ELECTRON MICROSCOPY.

RESULTS

Discussion

-pH, ACIDITY AND BUFFERING

*-IMMERSION OF TEETH AND NP- GHA IN WATER, AND
FRESH/FLAT/NEUTRAL PEPSI IN VITRO*

-SCANNING ELECTRON MICROSCOPY

Concluding Remarks

3.1) Abstract

INTRODUCTION: Recently, global pop-drink consumption has increased; however acid contents of pop-beverages and their effects on teeth are presumed innocuous.

Aim: To: (i) Assess acidity and buffering capacity *in vitro* of de-ionized water (DIW), Gassed, de-gassed (Flat-) and Neutralized Pop-cola (GFN-P). (ii) Compare tooth and non-porous granular hydroxyapatite (NP- GHA) erosion exposed to water and gassy/flat/neutralized pop-cola (GFN-P), (iii) Obtain and examine scanning electron *microscopic effects* on teeth and NP- GHA resulting from exposure to de-ionized water and GFN-P.

Materials and Methods: (i) pH and buffering capacities of DIW (Aquafina) and GFN-P (Pepsi-Cola) were measured by using a pH-Mettler Automatic Titrator, with weak solutions of Sodium Hydroxide (ml 0.5M NaOH). (ii) As controls calcium and phosphorus contents of DIW and GFN-P were measured with standardized chemical analytical methods. Dissolution of calcium and phosphorous from enamel and NP- GHA was assessed after 60 second immersion in the liquids. (iii) Before and after exposure to DIW and GFN-P, enamel and NP- GHA surfaces were examined using scanning electron microscope (SEM)

Results: (i) *In vitro* acid comparisons between DIW and GFN-P reveal highly significant difference in pH ($p < 0.05$), but statistically not significant differences in pH of gassy and flat Pepsi ($p > 0.05$). A highly significant ($p < 0.05$) difference in buffering capacities to pH 7, between gassy and flat Pepsi are reported. (ii) Bathing enamel and NP- GHA *in vitro* with gassy and flat Pepsi liquids caused marked mordant leaching of calcium and phosphorus. Yet with DIW and neutralized Pepsi, although both enamel and NP- GHA showed positive erosion, enamel showed least corrosion.

(iii) SEM shows corrosive erosion by Pepsi with both gassy and flat states, and confirmed scaled erosive damage recorded after soaking. **Conclusions:** (i) Gassy/degassed Pepsi acid activity are both below the critical pH 5.5 for chemical dissolution, with high buffering capacities *in vitro*; (ii) calcium and phosphorus are leached out of enamel and NP- GHA after bathing in gassy or degassed Pepsi; (iii) SEM evidence reveals no differences between gassy or degassed Pepsi and both cause erosion.

Key Words: Acid, Buffering, Calcium, Fresh Pepsi, Flat Pepsi, Neutral Pepsi, Erosion, Teeth, Hydroxyapatite.

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Érosion de l'émail et de l'hydroxyapatite

par de l'eau déionisée, et du cola gazéifié, dégazéifié et neutralisé

Observations in vitro et par microscopie électronique

3.1(A) : Résumé

INTRODUCTION : Récemment, la consommation globale de boissons gazeuses a augmenté. Cependant, le contenu acide de ces boissons et son effet sur les dents sont présumés inoffensifs.

Objectif : (i) Mesurer l'acidité et le pouvoir tampon *in vitro* de l'eau déionisée (DIW) et du cola gazéifié, dégazéifié (éventé) et neutralisé (GFN-P). (ii) Comparer l'érosion des dents et de l'hydroxyapatite granulaire non poreuse (NP- GHA) lorsqu'on les expose à de l'eau et à du cola gazéifié, éventé et neutralisé (GFN-P), (iii) obtenir et examiner, par *microscopie électronique* à balayage, les *effets* sur les dents et sur la NP- GHA résultant de l'exposition à de l'eau déionisée et au GFN-P. **Matériel et**

méthodes : (i) Le pH et les pouvoirs tampons de DIW (Aquafina) et de GFN-P (Pepsi-Cola) ont été mesurés à l'aide d'un pH-mètre et d'un titrimètre automatique de Mettler, avec une solution faible d'hydroxyde de sodium (ml NaOH 0.5 M). (ii) À titre de contrôle, on a mesuré la teneur en calcium et en phosphore de DIW et de GFN-P au moyen de méthodes normalisées de chimie analytique. La dissolution du calcium et du phosphore de l'émail et de la NP- GHA a été mesurée après un trempage de 60 secondes dans les liquides. (iii) La surface de l'émail et de la NP- GHA ont été examinées au microscope électronique à balayage (MEB) avant et après l'exposition aux DIW et GFN-P. **Résultats** : (i) Dans les comparaisons entre

l'acide vitro DIW et le GFN-P révèlent très grande différence dans les pH ($p < 0,05$), mais les différences statistiquement non significative du pH du gazeuses et plates Pepsi ($p > 0,05$). (ii) Le trempage de l'émail et du NP- GHA *in vitro* dans le Pepsi gazéifié et éventé a entraîné la chute marquée du mordant contenu dans le calcium et le phosphore. Cependant, en présence de DIW et de Pepsi neutralisé, bien que l'émail et la NP- GHA ont subi une érosion, l'émail a montré moins de signes de corrosion. (iii) Le MEB montre que le Pepsi, tant gazéifié qu'éventé, a entraîné une érosion-corrosion et a confirmé les dégâts proportionnels notés après le trempage.

Conclusions : (i) L'activité acidifiante du Pepsi gazéifié et éventé se situe sous le seuil critique de pH 5.5 en matière de dissolution chimique, avec des pouvoirs tampons élevés *in vitro*; (ii) le calcium et le phosphore sont éliminés de l'émail et de la NP- GHA après le trempage dans du Pepsi gazéifié ou éventé; (iii) le MEB montre qu'il n'y a pas de différence entre le Pepsi gazéifié et éventé : les deux provoquent de l'érosion.

Mots clés : Acide, Tamponnage, Calcium, Pepsi frais, Pepsi éventé, Pepsi neutre, Érosion, Dents, Hydroxyapatite.

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3.2) Introduction

Consumption of carbonated acidic soft drinks, like pop-colas, has become a regular sustained dietary habit among many people. Despite awareness of associated risk factors such as obesity and dental decay, most ignore potential side effects of these drinks and slake thirsts with pop daily.(24). One noticeable and irreversible risk associated with these drinks is dental erosion (chemical dissolution of tooth minerals) (25). In all age groups, and especially among school children, severity and frequency of dental erosion from consumption of carbonated beverages, acidulated drinks and foods has increased (26). Besides dentin and cementum, which make up the body and roots of teeth, the major calcified tissue covering the crowns of teeth in vertebrates, is dental enamel. Enamel is the most highly mineralized and hardest substance in the human body (27). It covers the anatomical crown and supports the underlying dentin. Enamel is not pure hydroxyapatite, but is composed of (93-95) % of hydroxyapatite (HA), with 5-7% of water, and other organic materials such as carbonate, sodium, magnesium, potassium, chloride, zinc, lipids, fluoride (27-29). Enamel Hydroxyapatite (HA), is not in a pure form but, also contains considerable amounts of carbonate, sodium, magnesium and chloride, as well as a small quantity of fluoride (30). Surface enamel integrity resists demineralization and relies on functioning roles of dental biofilm, saliva and other preventive agents (30). Enamel of newly erupted teeth is permeable to soluble fluids which penetrate into subsurface enamel, and increases HA susceptibility to acid dissolution. As enamel undergoes post-eruption maturation, in the presence of saliva, hydroxyapatite becomes more fluoridated and becomes more acid resistant and permeability of enamel is reduced

(28;29). Besides fluoride, calcium, and phosphorus contents of saliva, other factors such as acellular base layer of protective proteins of enamel pellicle, and proteins (statherin, proline-rich glycoproteins, and mucinous proteins) derived from saliva bind to HA and preserve enamel integrity (30). Although enamel surface is saturated with calcium, phosphate, and protective proteins, chronic exposure to acidulated drinks (<pH5.5) results in diffusion of hydrogen ions of acid content of the drinks into the fluid in the pores surrounding HA crystals and finally resulted in de-calcification and de-phosphorylation of the subsurface enamel (25). “Demineralization can be reversed if there is adequate time between acidogenic challenges to allow for remineralisation to occur” (22;30;31). Only minor microscopic erosions may be seen on tooth surfaces after first exposure to pop-cola, but in the long term, collective erosive loss of calcified tooth material will be apparent and clinical erosion with all its associated symptoms and stigmata become evident.

3.3) Aim

In this study, the aim is to investigate:

[i] The acidity as pH and buffering capacity of Aquafina Water (control), and fresh/flat (gassy/degassed) Pepsi-Cola,

[ii] *In vitro* chemical dissolution comparison of enamel surfaces of freshly extracted unerupted [human enamel crowns of] teeth and non-porous granular hydroxyapatite (NP- GHA) in water and flat/fresh/neutralized Pepsi-Cola, and

[iii] Scanning electron *microscopic effects* on enamel surfaces, and non-porous granular hydroxyapatite (NP-GHA), Aquafina water, and flat/fresh/neutralized Pepsi-Cola.

3.4) Methodology

3.4.1 pH AND BUFFERING: Aquafina de-ionized water (control) and Pepsi-Cola were analyzed. Pepsi Cola was selected as being the most acid and typical of pop-colas. (See Fig 3-1) Standard aliquots of water and Pepsi-Cola were tested for pH and buffering capacity. The acidity as pH and buffering capacity of Aquafina water, fresh, flat, and neutral Pepsi-Cola were measured with an automated METTLER DL25 Titrator [AT] (32). Buffering capacities were assessed with 0.5 M NaOH solution, titrated to raise the measured sample pH up to pH 7. Each of three cans of Pepsi-Cola degassed to a ‘flat’ state by using degassifier (sensitizer). The degassifier is a device with vibrating heated water, into which a gassy drink in its container is placed. When activated the degassifier heats its water and physical vibratory energy is transmitted into the pop-liquid, and forces release of dissolved gas (CO₂) from the drink. Three fresh 60mls aliquots were collected directly from each selected drink (Water, Fresh/Flat Pepsi-Cola) and placed in separate polystyrene falcon test-tubes for measurements. For neutralized Pepsi-Cola, a fresh can was degassed as above, and 0.5M NaOH titrated into it until pH7 using the specified automatic titrator. Titration measures were repeated for each sample from different cans in the same batch. For each sample, derived from the groups of drinks as stated in the different forms of drink (water, and fresh/flat/neutralized Pepsi-Cola), three titration measurements were done. See results in the **figures 3-1 and 3-2** below.

3.4.1(A) AUTOMATIC TITRATOR [AT]:

An AT was used to determine pH and buffering capacities of the water, and fresh/flat/neutral Pepsi. For this analysis, the AT was used according to described methods by Larsen et al 1998 method (33), and measured initial pH of each drink [Figure 3-1] and buffering capacities up to pH 7 [Figure 3-2]. All measures were assessed at room temperature of 23°C. To ensure measurement accuracy and establish base line controls, the AT was calibrated each time before use, at pH 2, 4, 7 and 10, using standard buffer solutions for all levels. A 60 mL volume of the tested sample was then titrated using a 0.5M NaOH solution. Measures were repeated 3 times for each group of selected drinks; the means of these are reported. See results in the figures below. To maintain neutrality as to eliminate inter- and intra-operator bias, data was analyzed ‘blind’ by third party technicians unaware of the source of procured samples.

3.4.1(B) RESULTS AND STATISTICS:

See **figures below**. Details of statistical analyses and all p-values stated in the text reported in **Appendix IV**.

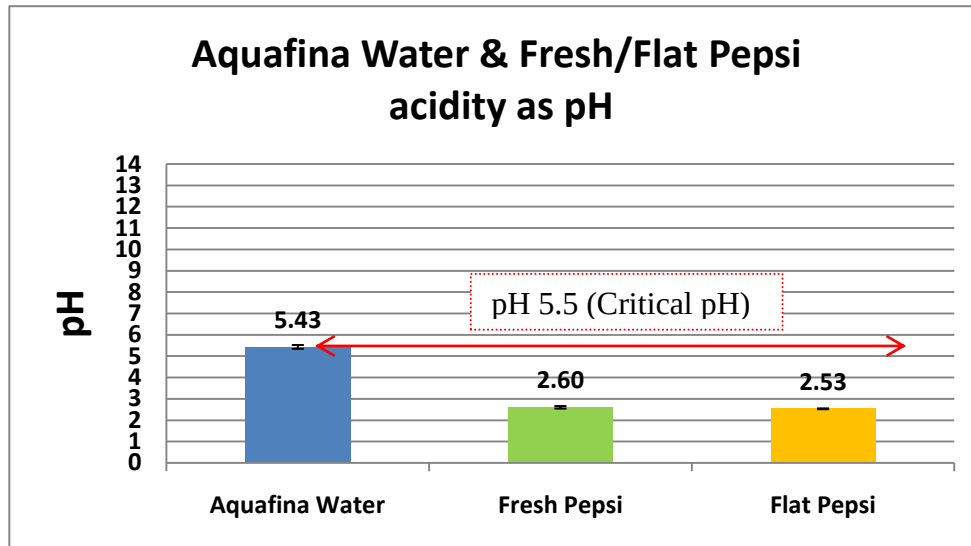


FIGURE 3-1: pH COMPARISON AMONG THE DRINKS: The pH levels of the Aquafina water is very close to critical pH value. Pepsi drinks (Fresh or Flat) are all significantly below the critical pH [pH5.5] of calcium-hydroxyapatite [$p < 0.05$]; but there is no significant differences between the acidity of fresh and flat Pepsi.

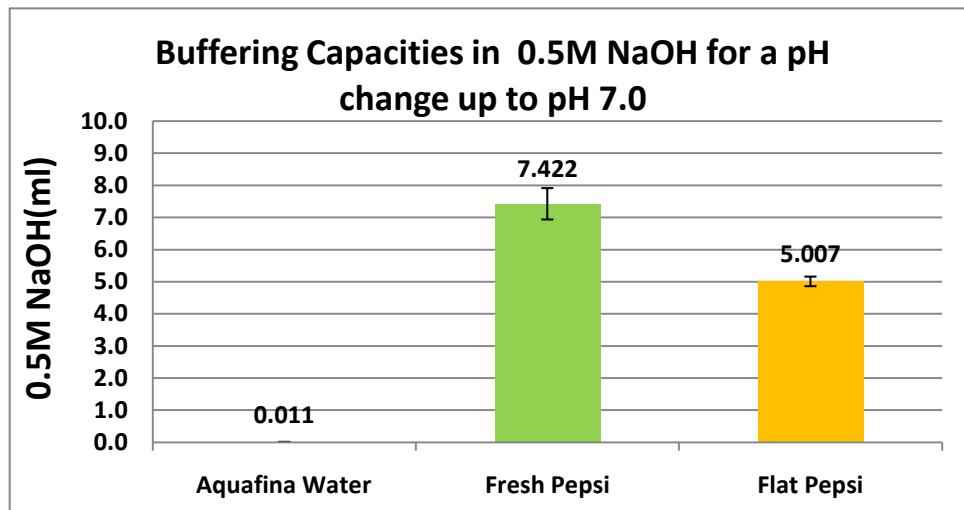


FIGURE 3-2: BUFFERING CAPACITIES OF SELECTED DRINKS UP TO pH7:

Buffering is different from neutralization. Buffering is the capacity to maintain pH at a given value and is assessed by comparing *volumes of alkali* to reach a specific pH. Aquafina water (control) has the lowest buffering capacity [0.011ml of 0.5 M NaOH] compared to Pepsi-cola. The green bar shows buffering capacities for change

from the fresh Pepsi initial pH up to pH 7 having the highest buffering capacity [7.422 ml of 0.5 M NaOH] while flat Pepsi has the lower buffering capacity [5.007ml of 0.5M NaOH]. Both fresh and flat Pepsi absorb the alkali, but vary in the amount of alkali to reach pH7. This is attributed to the acid formation of CO₂ gas, dissolving in water to form carbonic acid. Once the CO₂ is removed the hydrogen ion donor mechanism is removed and less NaOH is required to neutralize the remaining cola.

3.4.2 IMMERSION OF TEETH AND NP- GHA IN WATER, AND FRESH/FLAT /NEUTRAL PEPSI:

The four different drinks mentioned [(i) Aquafina water, (ii) Fresh (iii) Flat (iv) and Neutralized Pepsi] were tested. Six samples of *granular hydroxyapatite* and *fresh unerupted (from surgical removed impacted wisdom teeth) human teeth* for each drink were used to rinse with 10ml aliquots of water and fresh/flat/neutralized Pepsi for 60 seconds for each sample. In the case of flat Pepsi-Cola, a degassifier was used for 30 minutes to completely remove any gas content of the drink. For neutralized Pepsi, the Automatic Titrator set to pH 7 and Pepsi titrated by 0.5 M NaOH to exactly neutral pH value [pH 7.0]. Every sample was analyzed using standardized analytical chemistry methods [**Figure 3-3**] (34). The Pepsi from source and post NP-GHA immersion were analyzed for calcium, and phosphorous contents using *Inductively Coupled Plasma with Optical Emission Spectroscopy [ICP-OES]* (34) [**See Appendix II**]. For our analysis, concentrations of calcium and the major ions of interest as phosphate are reported here (35). To eliminate inter- and intra-

operator bias, results for the data was checked and confirmed ‘blind’ by third party technicians, unaware of the source of procured samples or the experimental design.

3.4.2(A) RESULTS AND STATISTICS:

One-way Analysis of Variance (ANOVA) was used to compare the mean of Calcium and phosphorus by group (Aquafina Water, Pepsi with gas, Pepsi without gas, neutral Pepsi) for both non-porous granular hydroxyapatite and teeth **[Figures 3-3 to 3-6]**. In order to identify group differences *Post-hoc pairwise comparisons* of means were performed when the overall *F-test* concluded for a significant difference among the groups. P-values for post-hoc comparisons were adjusted for multiple testing using the bootstrap with 5000 samples(36). Assumptions of homogeneity of error variances and normality of the observations within each group were checked by residual analysis. In case of evidence of non-normality the outcome variable was transformed by natural logarithm (to remove positive skewness). In phosphorus analysis **[Figure 3-4 & 3-6]**, an ANOVA test performed for all groups except for aquafina Water as its values are very low compared to other groups. The ANOVA test showed an obvious significance difference among the groups. **[Appendix IV, Table 01& 02]**

Analysis reveals consistent significant [$p < 0.05$] increases in calcium leached from NP-GHA and from crowns of teeth after immersion into all drinks tested. The ICP-OES measures were compared in both test-samples (NP-GHA and teeth) individually and they show significant calcium concentration loss **[Figures 3-3 and 3-5]** and slight phosphorus concentration variations **[Figures 3-4 and 3-6]** after immersion in the drinks. Comparison of ICP-OES measures (Teeth and NP-GHA

exposed) between the two, the samples NP- GHA shows more de-calcification than teeth [Figures 3-3 and 3-5]. In both, there is evidence of slight de-phosphorylation in the Pepsi-Cola drinks [Figures 3-4 and 3-6].

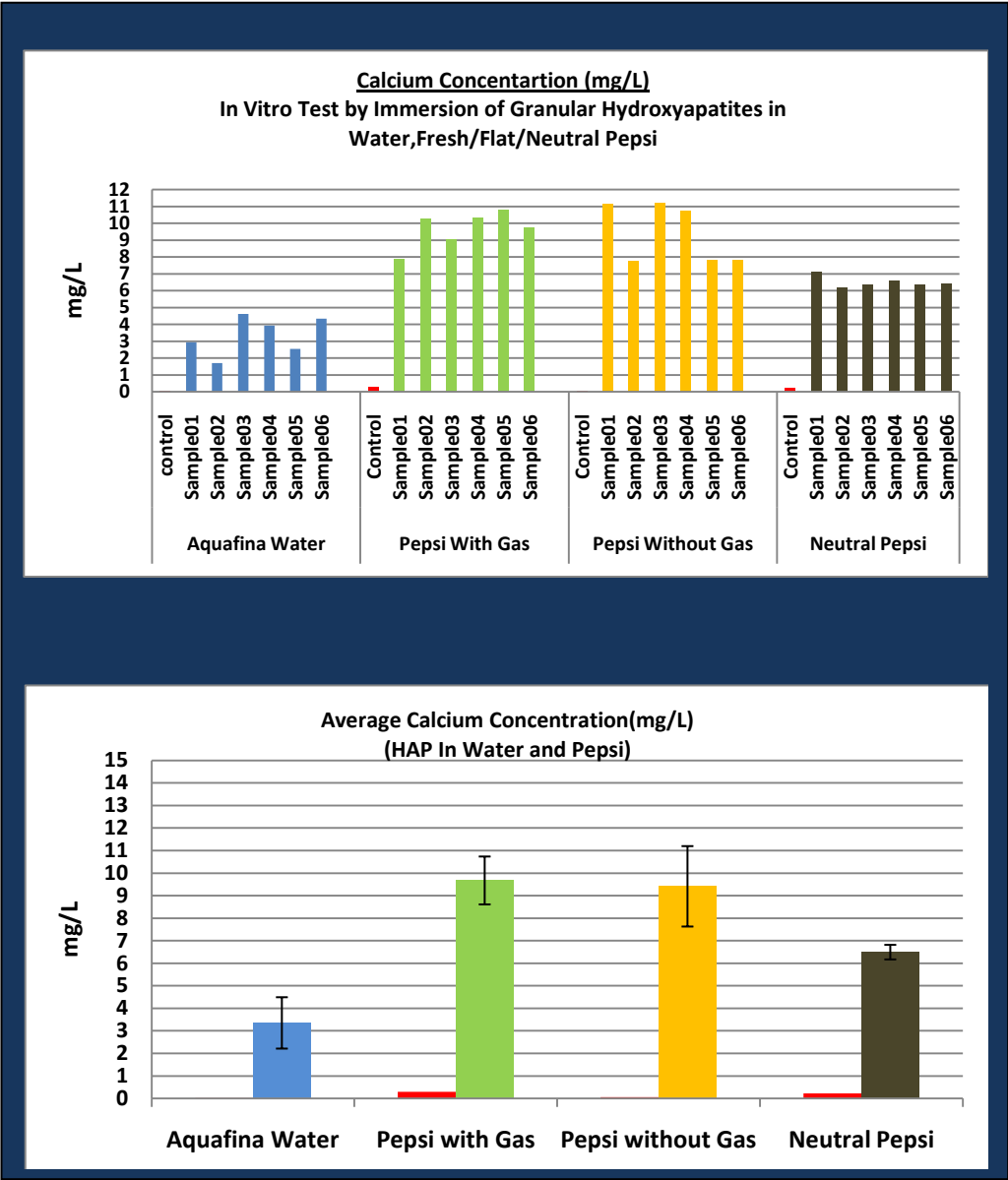


FIGURE 3-3: IONIC CALCIUM CONCENTRATION MEASURES: Before and after immersion of one crystal ($200\text{mg} \pm 0.001$) NP-GHA in each of 10mls of the drinks. One sample of each drinks used as controls [Red bars]. Negligible (undetectable)

amounts of calcium exist in the controls. Each NP-GHA sample was soaked for 60 seconds, after which the remaining NP-GHA was removed. After the NP-GHA immersion, the remaining liquid samples were acidified with 5ml HNO_3 to denature any organic components; the resultant mix was diluted to reach 20 ml with distilled water. This gives enough volume for testing with ICP-OES, according to accepted chemistry analytic technique ICP-OES (12). Two hours later the liquid samples were measured for calcium from source [bottled water or can] and from those liquids immersed with NP- GHA crystals. The Ca^{++} content of each of the six samples, in every group varies within a narrow range. Calcium expressed as mg/L from the source and for test-drinks after immersion with NP-GHA reveal a significant increase [$p < 0.05$] of calcium, found in all the liquids tested (immersed with NP-GHA). The calcium content in the controls, shown as red bars, is negligible.

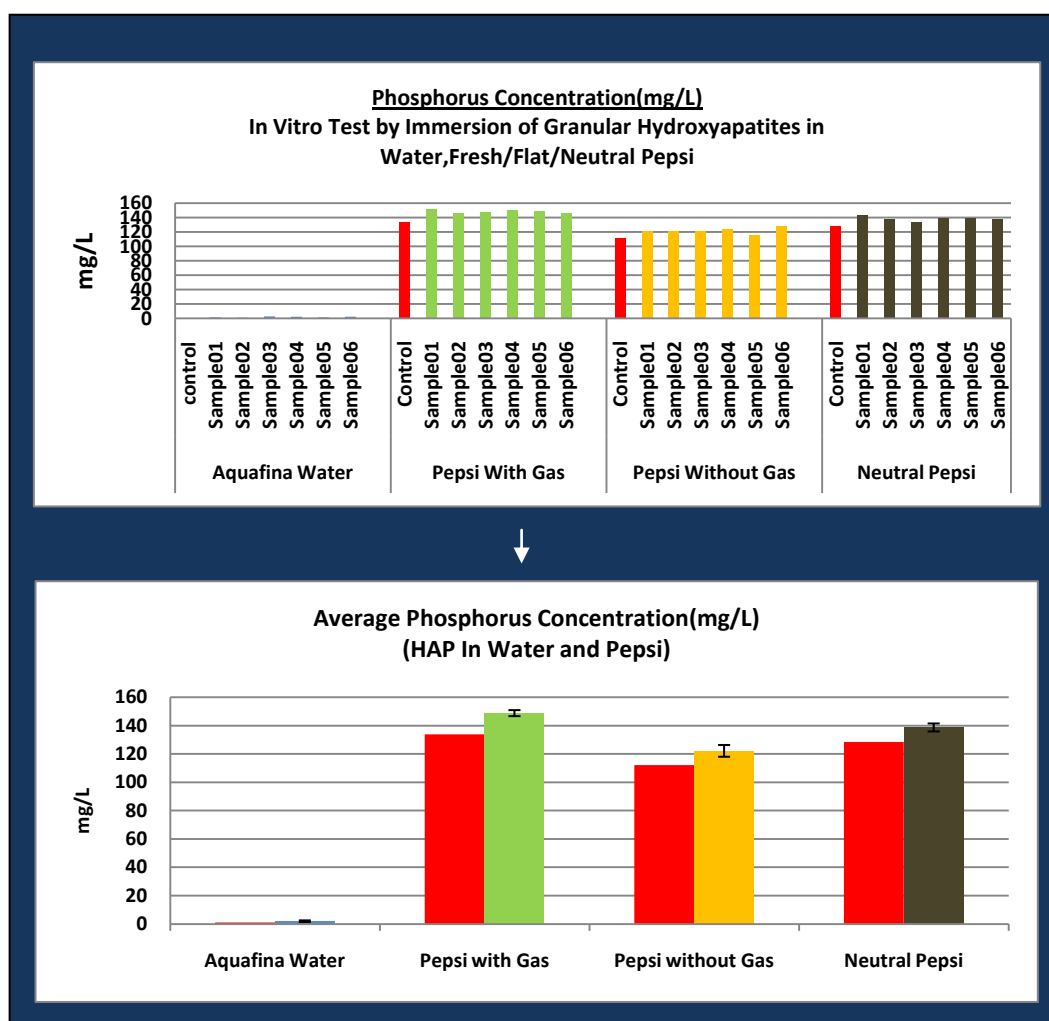


FIGURE 3-4: PHOSPHORUS CONCENTRATION MEASURES BEFORE AND AFTER IMMERSION OF NP-GHA IN THE DRINKS: Expressed as mg/L from the source [bottled water or can] and test-drinks. One sample of each drink used as a control (Red bars). NP-GHA Crystals immersed in the drinks (water-blue, Pepsi with gas-green, Pepsi without gas-yellow and neutralized Pepsi-brown bars). After the NP-GHA immersion, the remaining liquid samples were acidified with 5ml HNO_3 to denature any organic components; the resultant mix was diluted to reach 20 ml with distilled water. This gave enough volume for testing with ICP-OES, according to

accepted chemistry analytic technique ICP-OES (12). The liquids were tested for calcium and phosphorus simultaneously. Two hours later the liquid samples were measured for Phosphorous from source [bottled water or can] and from those liquids immersed with NP-GHA crystals. Higher phosphorus concentrations were detected in the liquids after NP-GHA immersion except for Aquafina water. Increase in phosphorus concentration is due to leaching of phosphorus ions out of NP-GHA crystals.

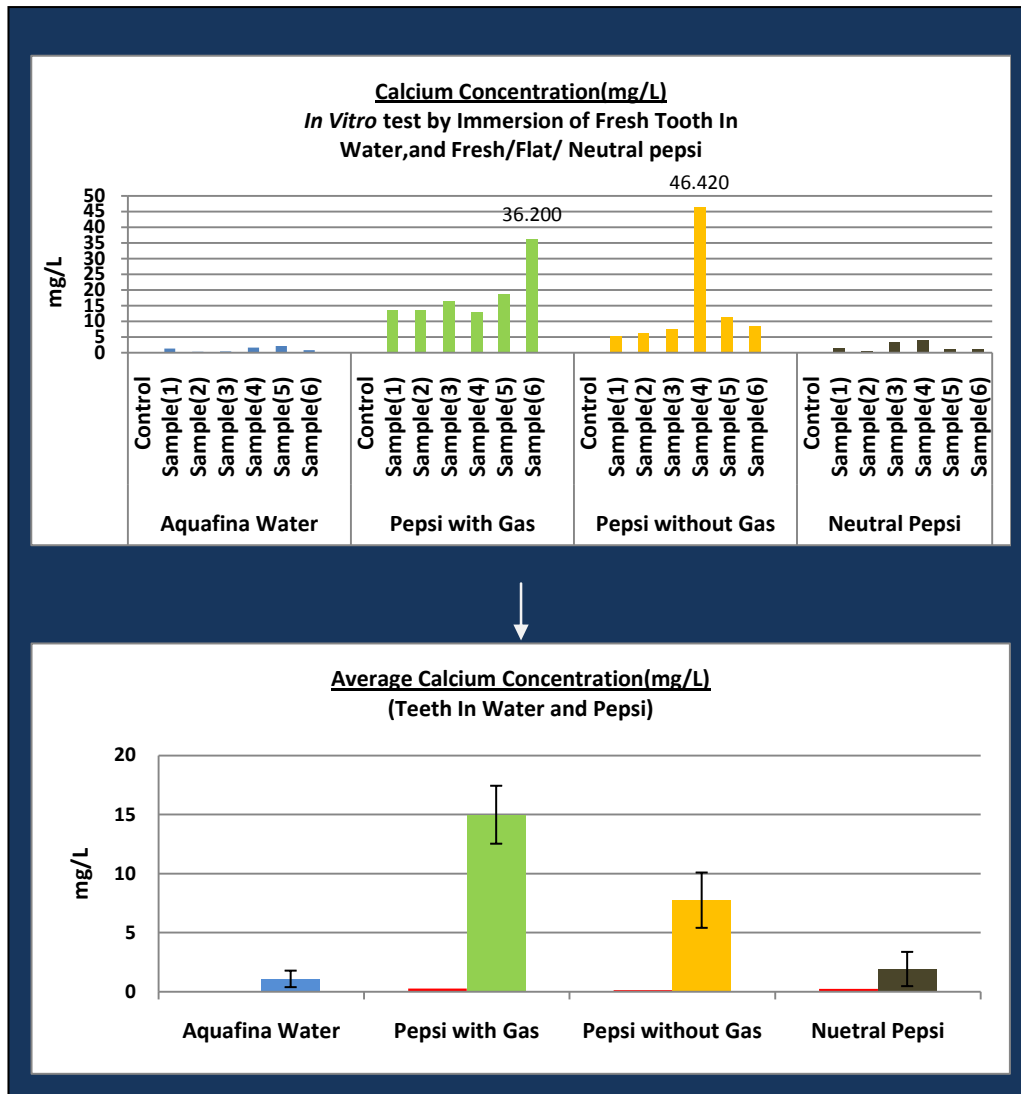


FIGURE 3-5: IONIC CALCIUM CONCENTRATION MEASURES: before and after immersing the enamel of a whole clinical crown, of one unerupted wisdom tooth, in each of 10ml of the drinks. Ten teeth from five patients were pooled (age range 19-22 years: 3M&2F); from those 10 teeth 6 were chosen by chance for testing. The roots were sealed off with beeswax at the ECJ. One sample of each drink was used as controls [Red bars]. Negligible (undetectable) amounts of calcium exist in the controls. Each tooth-crown sample was soaked for 60 seconds, after which the

remaining tooth was removed. After the tooth-crown immersion, the remaining liquid samples were acidified with 5ml HNO_3 to denature any organic components; the resultant mix was diluted to reach 20 ml with distilled water. This gave enough volume for testing with ICP-OES, according to accepted chemistry analytic technique ICP-OES (12). Two hours later the liquid samples were measured for calcium from source [bottled water or can] and from those liquids in which the crowns were immersed for 60 seconds. The Ca^{++} content of each of the six samples, in every group varies as the teeth were derived from different patients. Calcium expressed as mg/L from the source and for test-drinks after immersion with a tooth, reveal a significant increase [$p < 0.05$] of calcium, found in the gassy and degassed Pepsi-Cola tested. Very small amounts of calcium leached out from the teeth in the water and neutralized Pepsi; the amounts were not significantly different between these two.

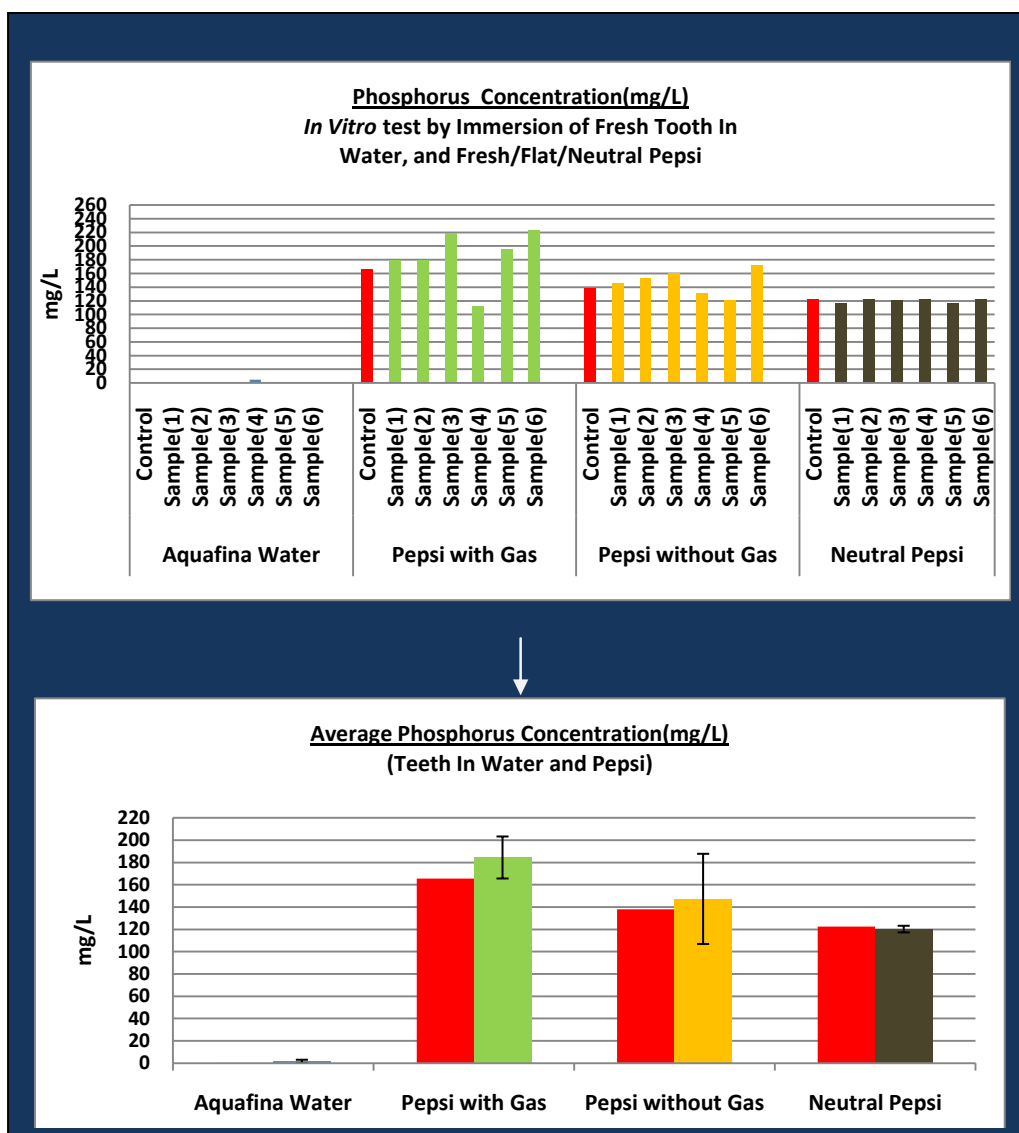


FIGURE 3-6: PHOSPHORUS CONCENTRATION MEASURES BEFORE AND AFTER IMMERSION OF TEETH IN THE DRINKS: Expressed as mg/L from the source [bottled water or can] and test-drinks. One sample of each drinks used as controls (red bars). The same *fresh human unerupted wisdom teeth used for calcium detection were used for simultaneous phosphate detection*. The phosphorus content of control probes obtained from each drinks were measured with ICP-OES and the results vary. This higher phosphorus concentration was detected after immersion of teeth into

drinks except for Aquafina water and neutral Pepsi-Cola. Increases in Phosphorus concentration is due to leaching of phosphorus ions out of teeth crystals.

3.4.3 SCANNING ELECTRON MICROSCOPY:

Biosynthetic granular hydroxyapatite (non-porous) and fresh pristine teeth extracted by professionally qualified clinicians were used. Each selected drink was tested on surfaces of NP-GHA crystals and enamel surfaces of the teeth; this was repeated six times for each selected drink, with only two samples [from each selected drink] being selected by chance for SEM. All areas of focused interest were examined using scanning electron microscopy with variable-pressure scanning electron microscopy VP-SEM (37). Resolution was at 700X and 1500X, with accelerating voltage 20-25 kV used, to assess fracture patterns and morphology. Immediately after scanning controls, each sample was immersed in one of the drinks for total of 60s and the *same location* was scanned and recorded. Identical site-surfaces were scrutinised and the *same native precise areas* were examined. No sputter or coating enhancement with metal or gold plasma was used. Image analysis of morphology, properties and characteristics [cracks, openings and changes of identifiable shapes] were done with microscopic measuring scales.

3.4.3(A) RESULTS [SEE FIGURES 3-7 TO 3-22 BELOW]:

Ultra-scaled measures [700X & 1500X] revealed losses of hard tissue from erosion. Initial erosion was seen after exposure to the drinks, as chemical loss of calcium causes surface crenellations to develop. After 60s cola exposure, soft surface crenellations developed. Here only two samples for each drink were chosen randomly for analysis as all others showed similar results.

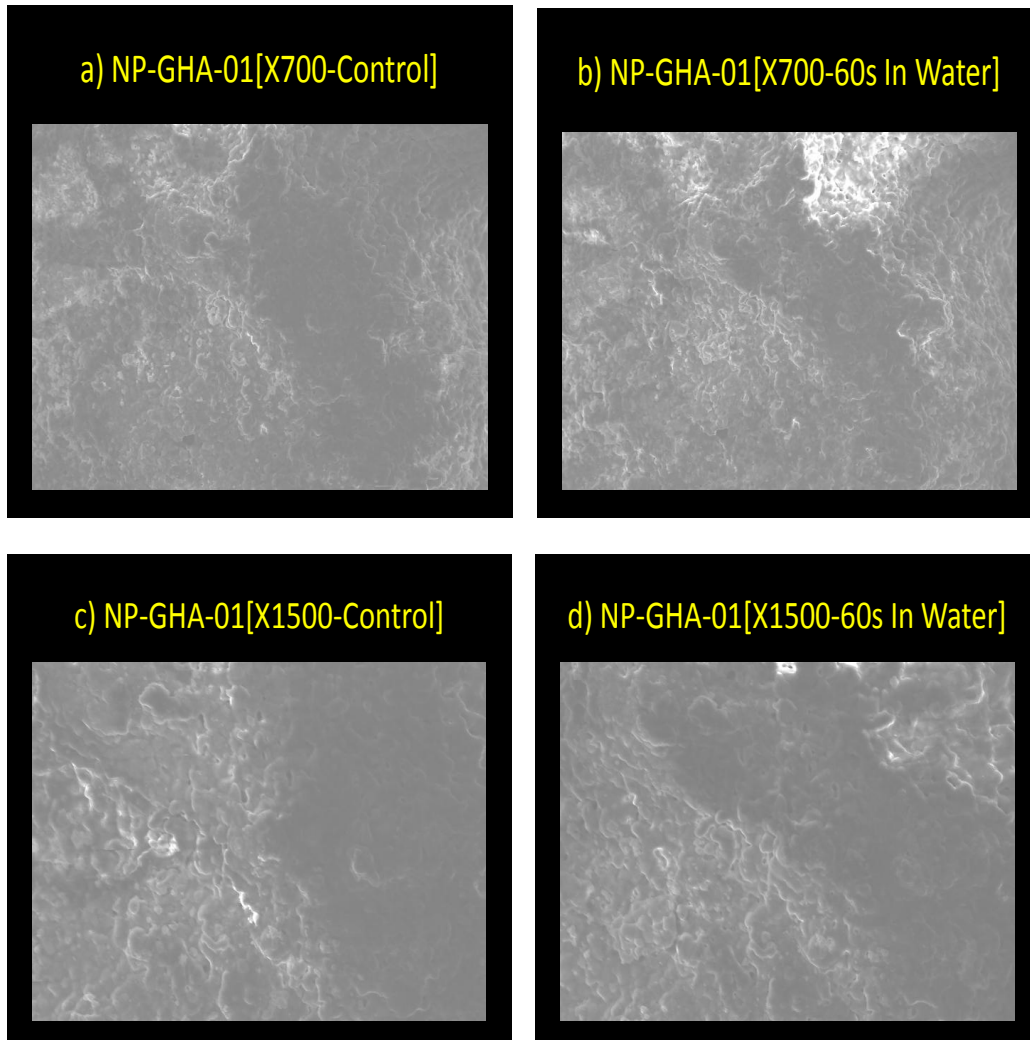


Figure 3-7(Sample01): *Non- porous granular hydroxyapatite & Aquafina water.*

The selected areas show minor irrelevant surface changes when comparing the initial [Figures 3-7 (a, c)] with the subsequent presentations [figures 3-7 (b, d)] after 60s exposure to water.

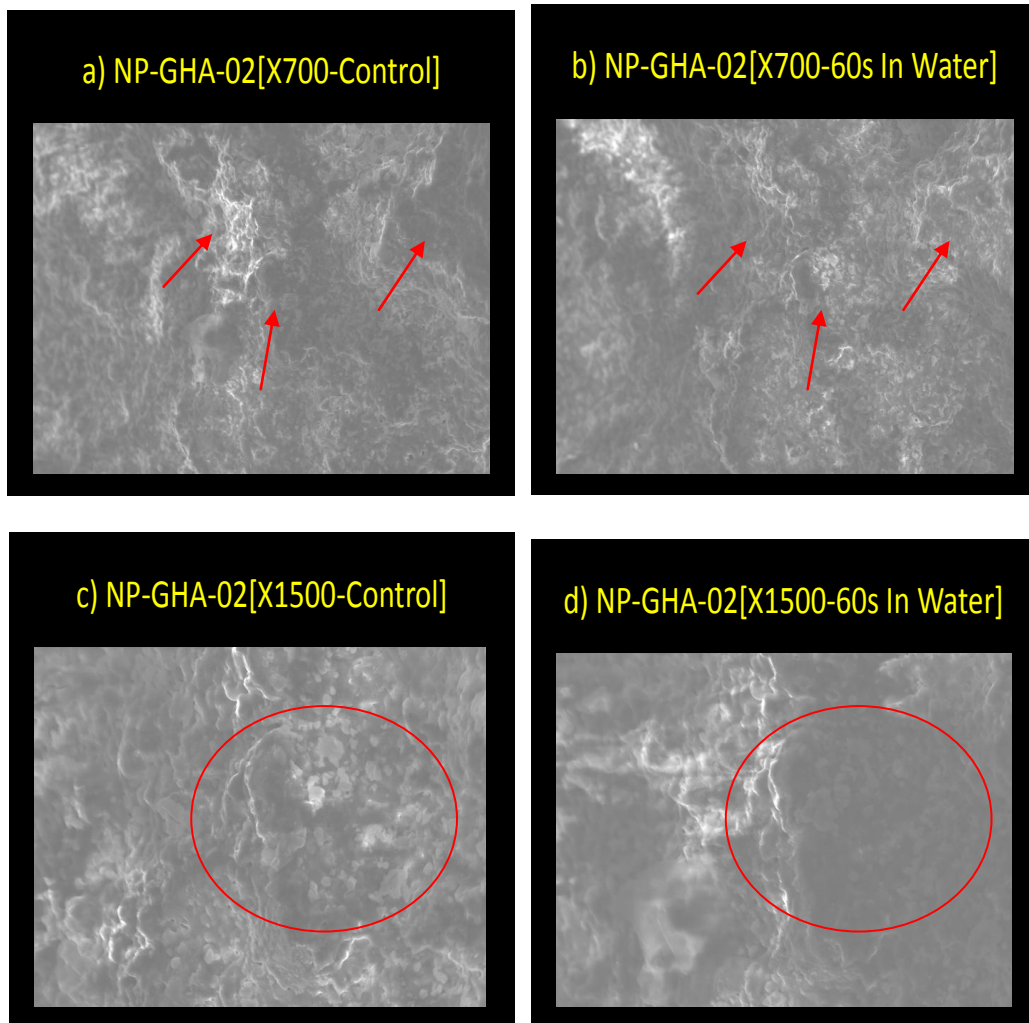


Figure 3-8 (Sample02): *Non-porous granular hydroxyapatite & Aquafina water.*

Areas, indicated with red arrows and ovals, show minor surface changes from calcium loss when comparing **figures 3-8 (b, d)** to controls in **Figures 3-8 (a, c)**.

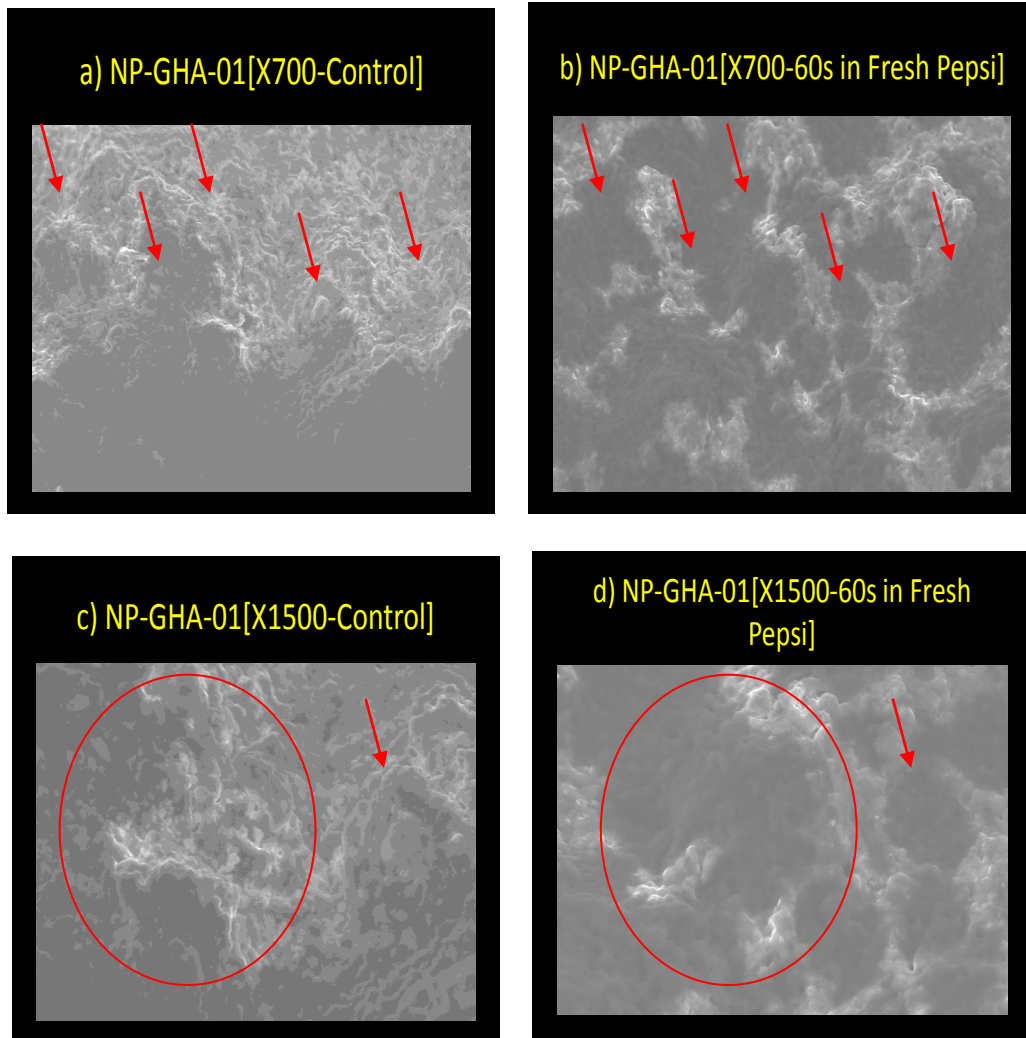


Figure 3-9(Sample01): *Non- porous granular hydroxyapatite & Fresh Pepsi.*

Red arrows and oval outlines show deep chemical dissolution of all exposed superficial layers in the same areas for NP-GHA, and indicate obvious locations of change from chemical dissolution when comparing **Figures 3-9 (b, d)** to controls in **Figures 3-9 (a, c)**. These SEM results are consistent with the calcium dissolution from exposure to fresh Pepsi in **Figure 3-3**.

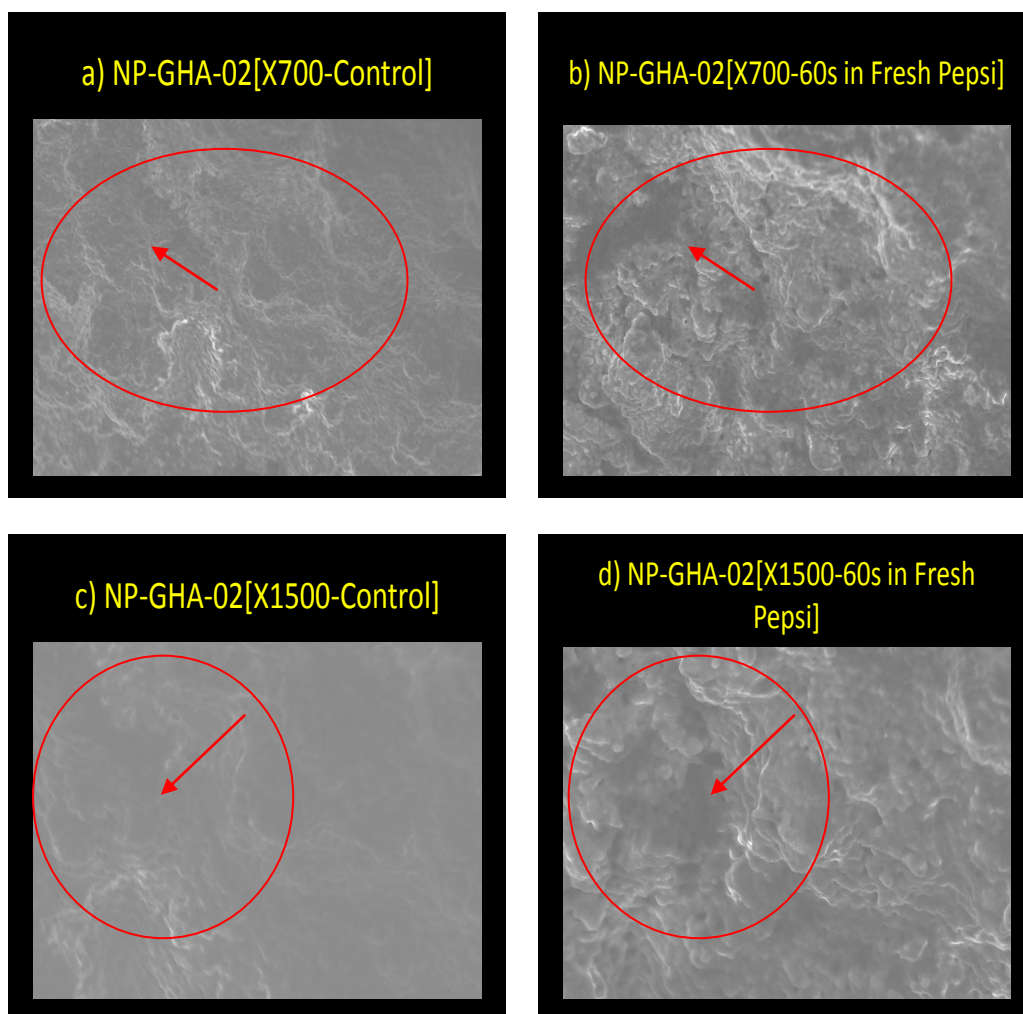


Figure 3-10 (Sample02): *Non- porous granular hydroxyapatite & Fresh Pepsi.*

Red arrows and oval outlines show areas with deep chemical dissolution of all exposed superficial layers in the same locations for NP-GHA. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-10 (b, d)** to controls in **Figures 3-10 (a, c)**. These SEM results are consistent with the calcium dissolution from exposure to fresh Pepsi in **Figure 3-3**.

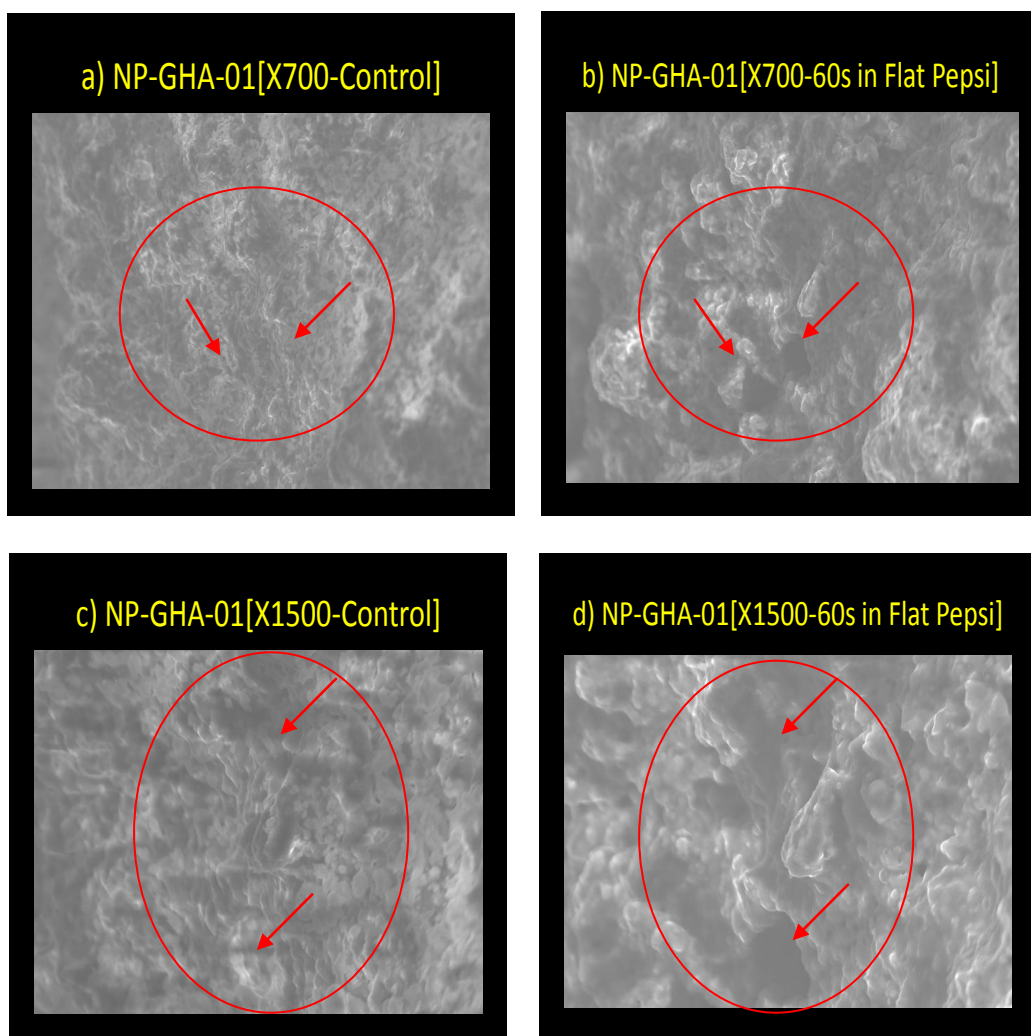


Figure 3-11(Sample01): *Non- porous granular hydroxyapatite & Flat Pepsi.*

Red arrows and oval outlines show deep chemical dissolution of all exposed superficial layers in the same areas for NP-GHA. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-11 (b, d)** to controls in **Figures 3-11 (a, c)**. These SEM results are consistent with the calcium dissolution from exposure to flat Pepsi in **Figure 3-3**.

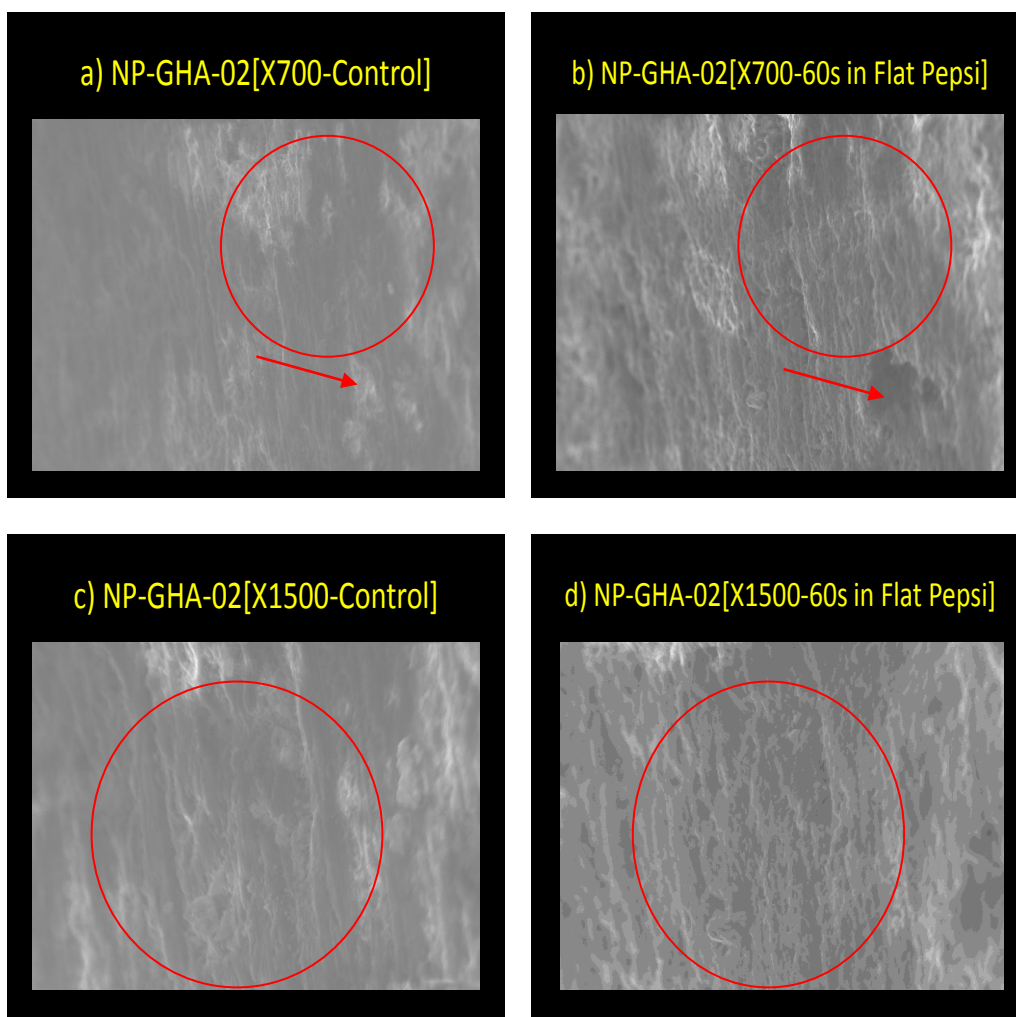


Figure 3-12(Sample02): *Non- porous granular hydroxyapatite & Flat Pepsi.*

Red arrows and oval outlines show deep chemical dissolution of all exposed superficial layers in the same areas for NP-GHA. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-12 (b, d)** to controls in **Figures 3-12 (a, c)**. These SEM results are consistent with the calcium dissolution from exposure to flat Pepsi in **Figure 3-3**.

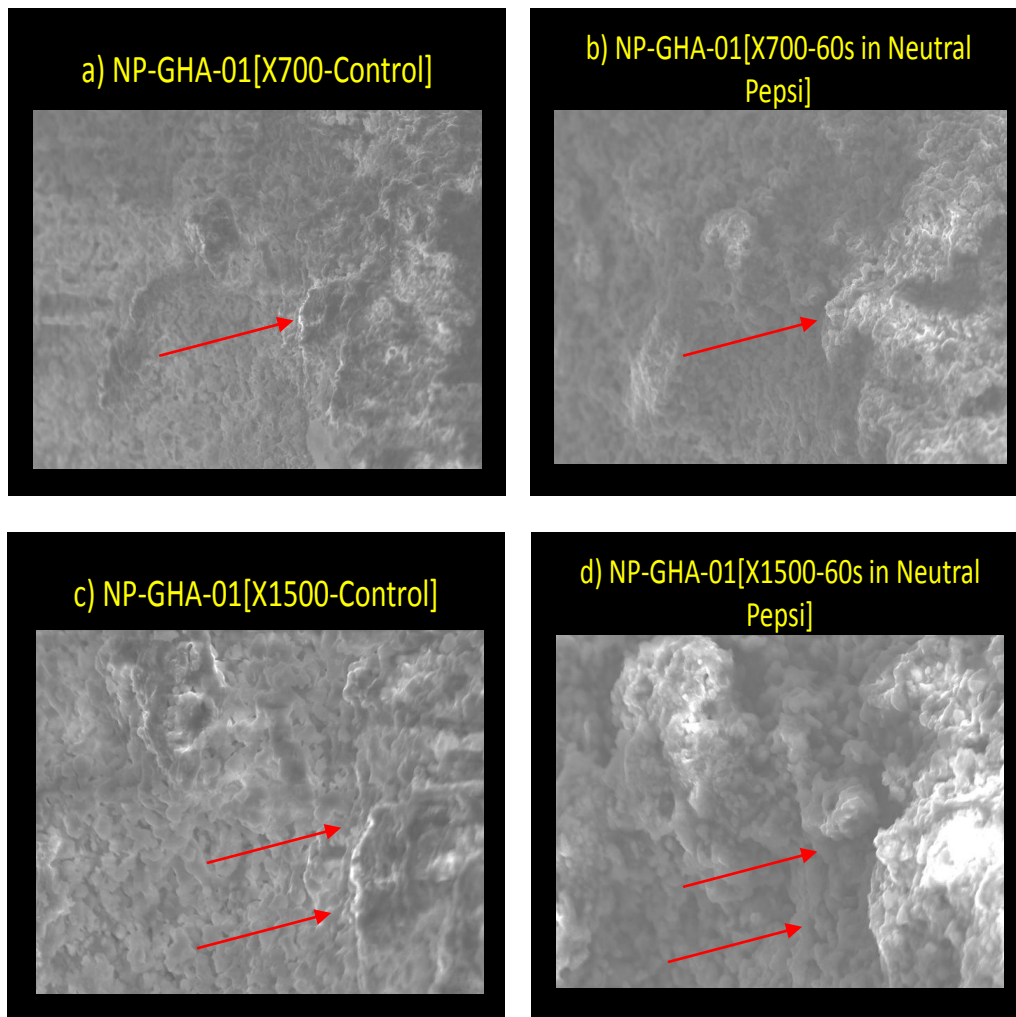


Figure 3-13(Sample01): *Non- porous granular hydroxyapatite & Neutral Pepsi.*

At neutral pH 7, the constituents of Pepsi still have a surface effect. The red arrows indicate a discrete structure. When comparing **Figures 3-13 (a, c)** with **Figures 3-13 (b, d)**, the structure diminishes in definition, loses sharp edges, becomes rounded and partially disappears. This is interpreted as loss of constituent substance due to dissolution from exposure to neutral Pepsi.

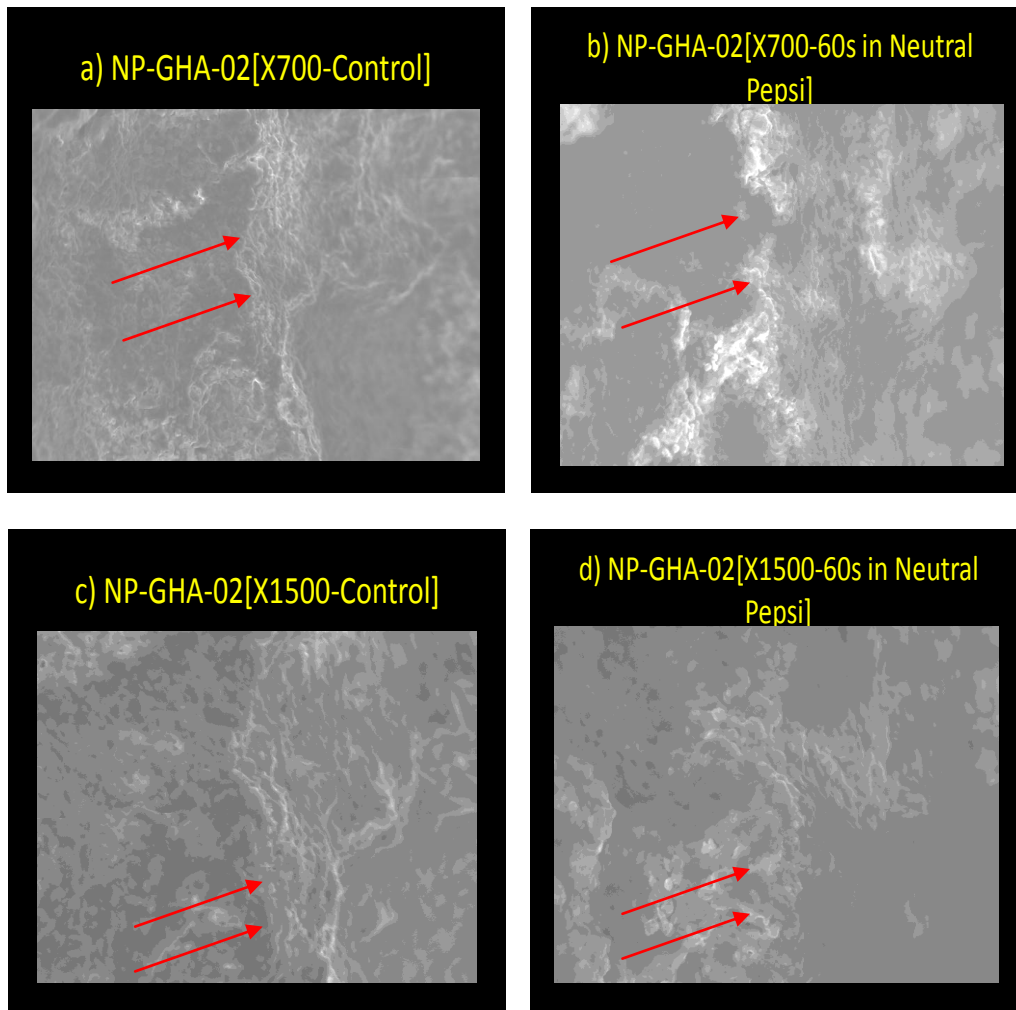


Figure 3-14(Sample02): *Non- porous granular hydroxyapatite & Neutral Pepsi.*

At neutral pH 7, the constituents of Pepsi still have a surface effect. The red arrows indicate a discrete structure. When comparing **Figures 3-14 (a, c)** with **Figures 3-14(b, d)**, the structure decreases definition, loses sharp outlines, becomes bolder in shape and some shapes disappear. This is interpreted as loss of constituent substance due to dissolution from exposure to neutral Pepsi.

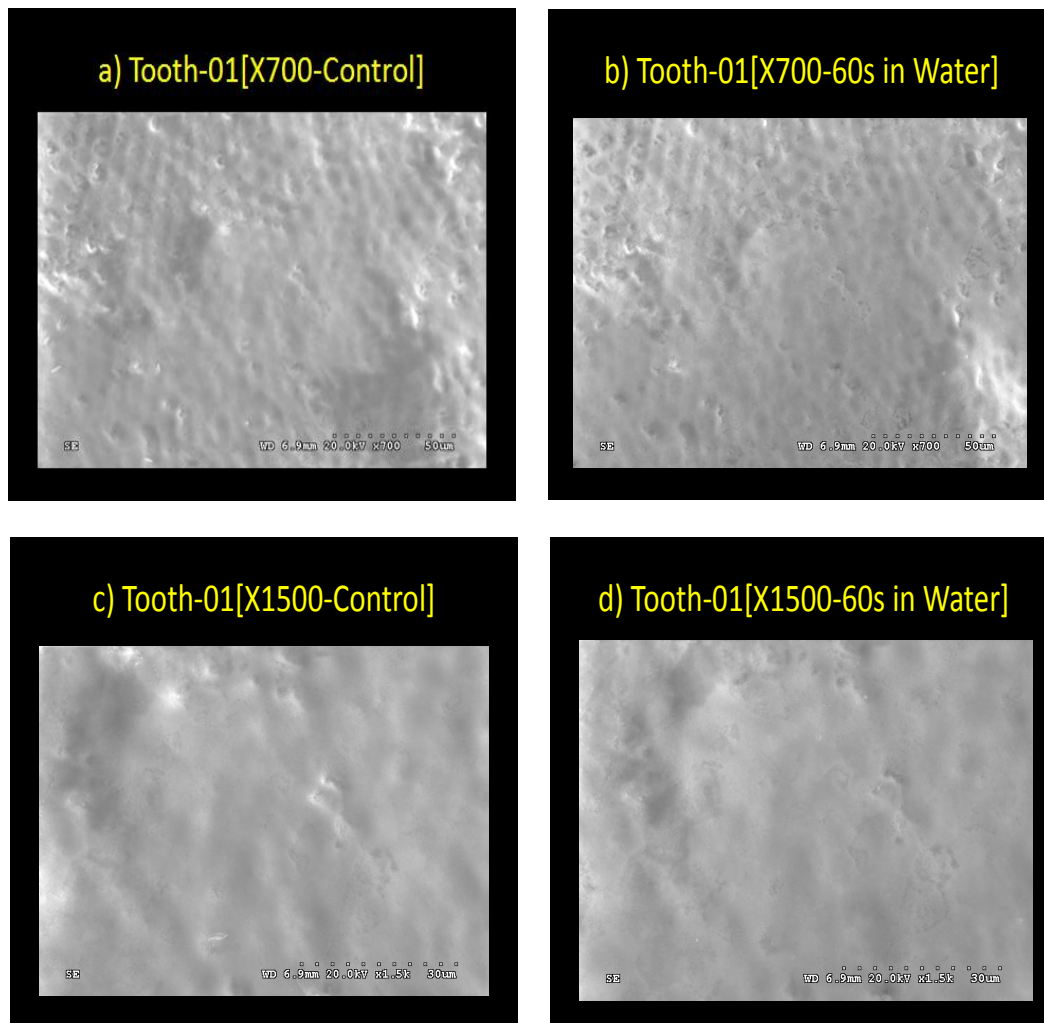


Figure 3-15(Sample01): *Tooth & Aquafina Water.*

When comparing **Figures 3-15 (a, c)** with **Figures 3-15(b, d)**, exposure of enamel reflects minimal changes. The differences are irrelevant and are probably artifacts.

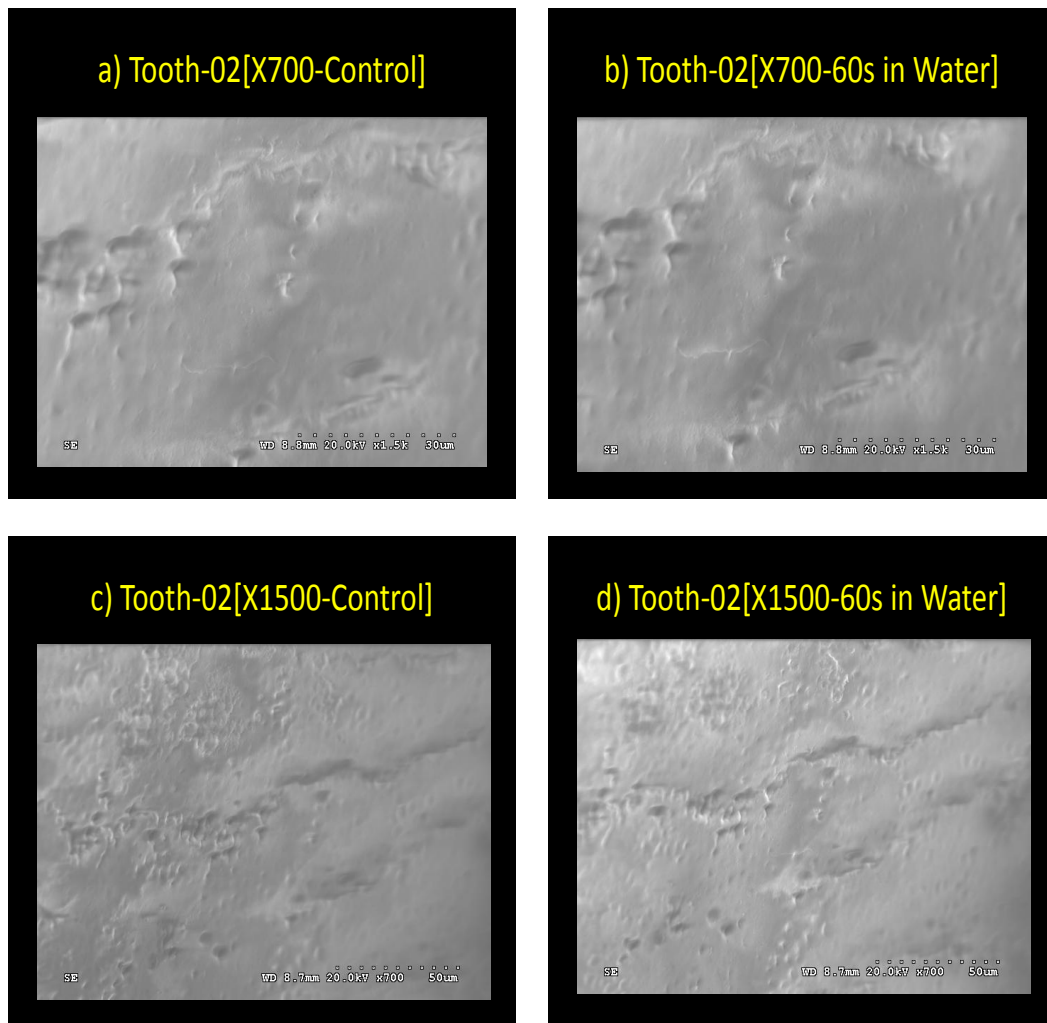


Figure 3-16(Sample02): *Tooth & Aquafina Water.*

When comparing **Figures 3-16 (a, c)** with **Figures 3-16(b, d)**, exposure of enamel reflects minimal changes. The differences are irrelevant and are probably artifacts.

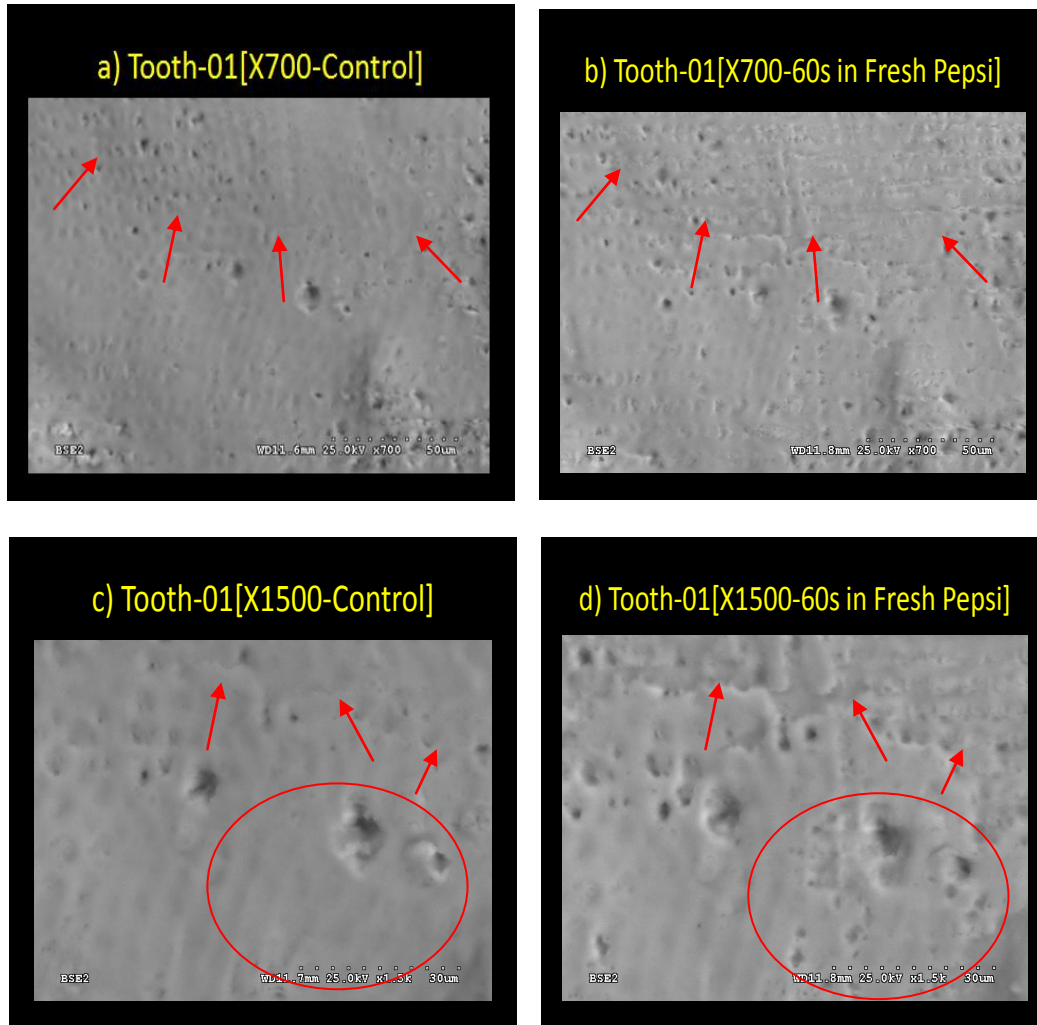


Figure 3-17(Sample01): *Tooth & Fresh Pepsi.*

Red arrows and oval outlines show deep chemical dissolution and Surface etching of all exposed superficial layers in the same areas in teeth. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-17 (b, d)** to controls in **Figures 3-17(a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

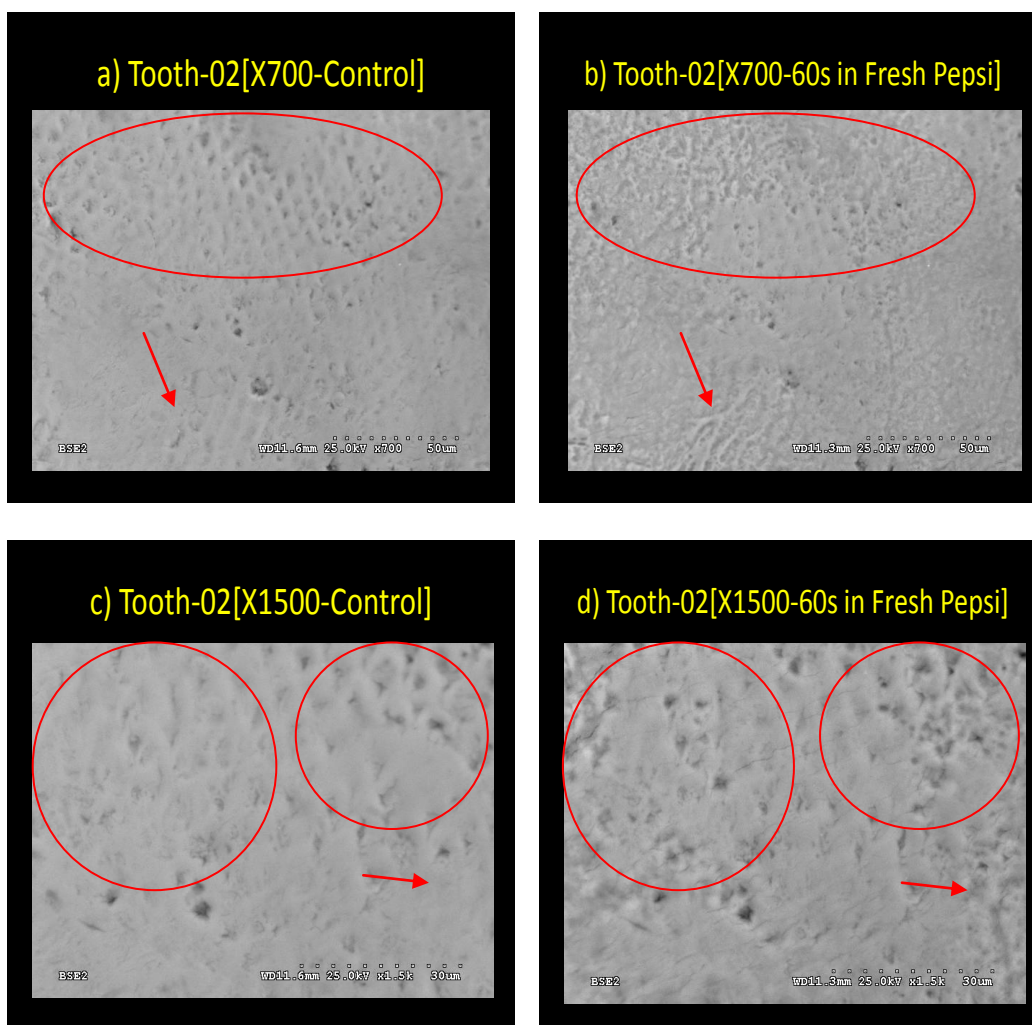


Figure 3-18(Sample02): *Tooth & Fresh Pepsi.*

Red arrows and oval outlines show deep chemical dissolution and Surface etching of all exposed superficial layers in the same areas in teeth. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-18 (b, d)** to controls in **Figures 3-18(a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

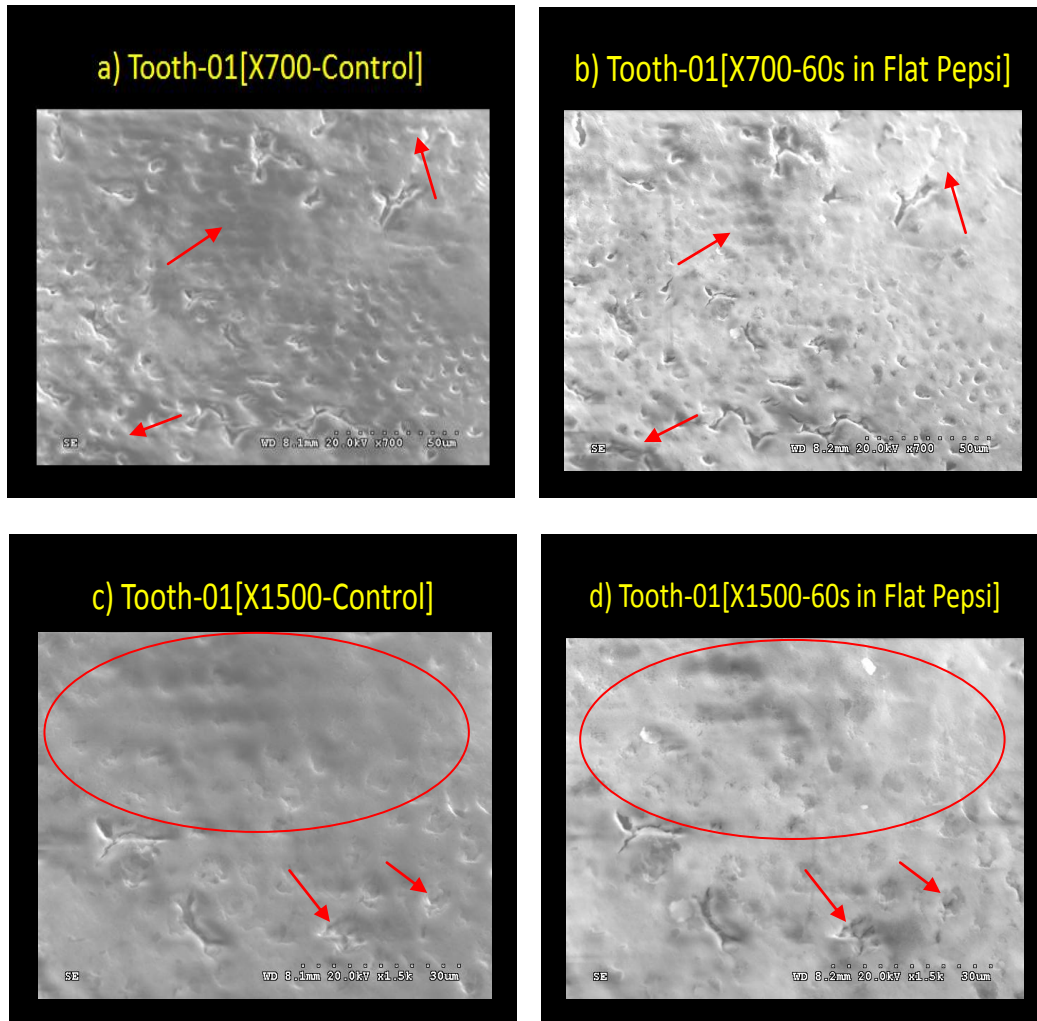


Figure 3-19(Sample01): *Tooth & Flat Pepsi.*

Red arrows and oval outlines show deep chemical dissolution and Surface etching of all exposed superficial layers in the same areas in teeth. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-19 (b, d)** to controls in **Figures 3-19(a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

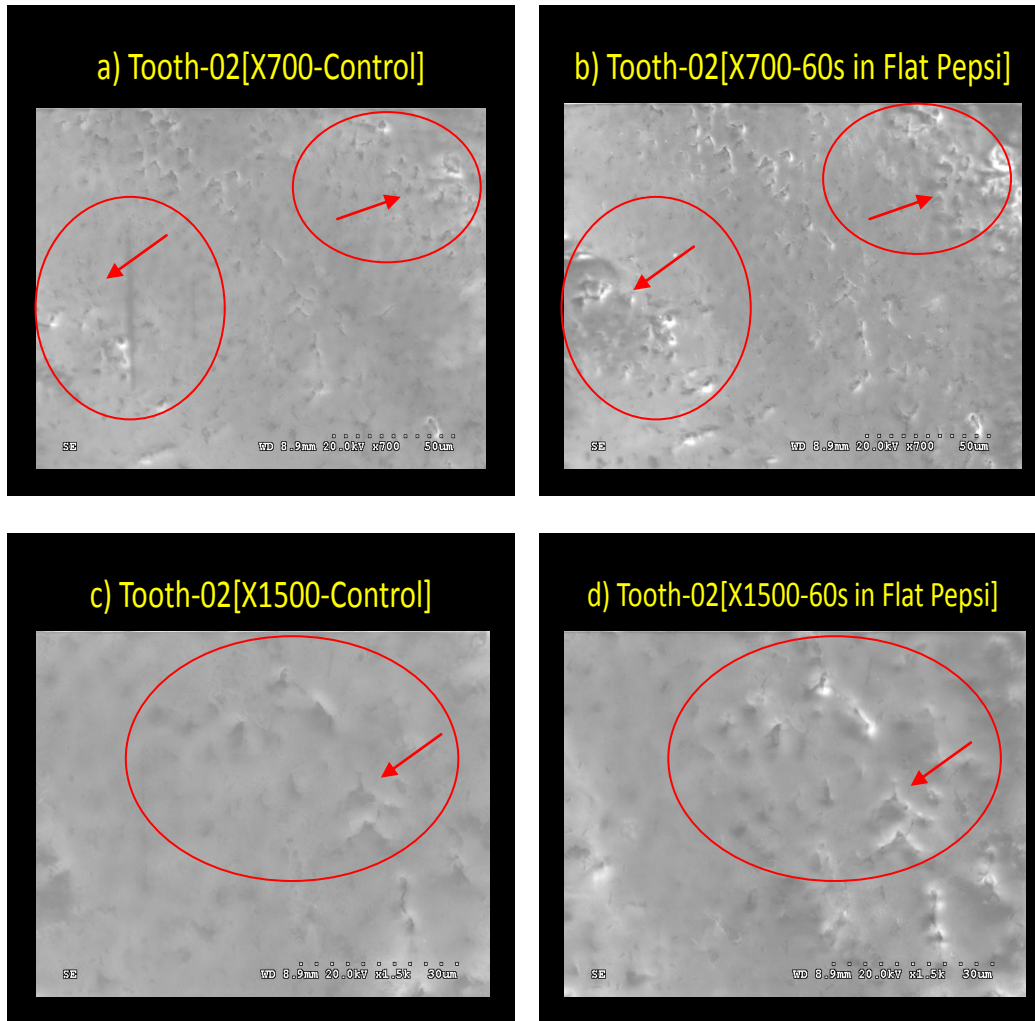


Figure 3-20(Sample02): *Tooth & Flat Pepsi.*

Red arrows and oval outlines show deep chemical dissolution and Surface etching of all exposed superficial layers in the same areas in teeth. Red arrows indicate obvious locations of change from chemical dissolution when comparing **Figures 3-20 (b, d)** to controls in **Figures 3-20(a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

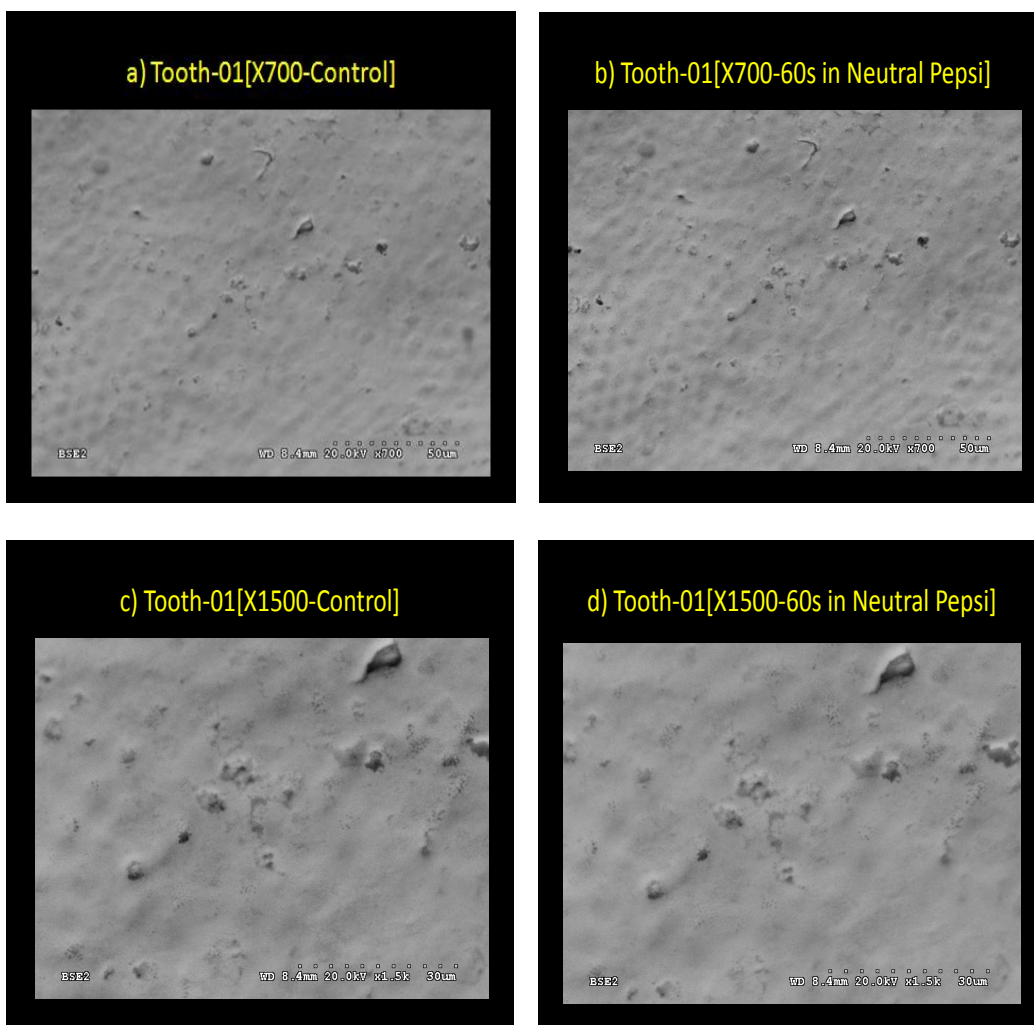


Figure 3-21(Sample01): *Tooth & Neutral Pepsi.*

Areas show minimal surface changes from exposure of teeth to neutral Pepsi when comparing **figures 3-21 (b, d)** to controls in **Figures 3-21 (a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

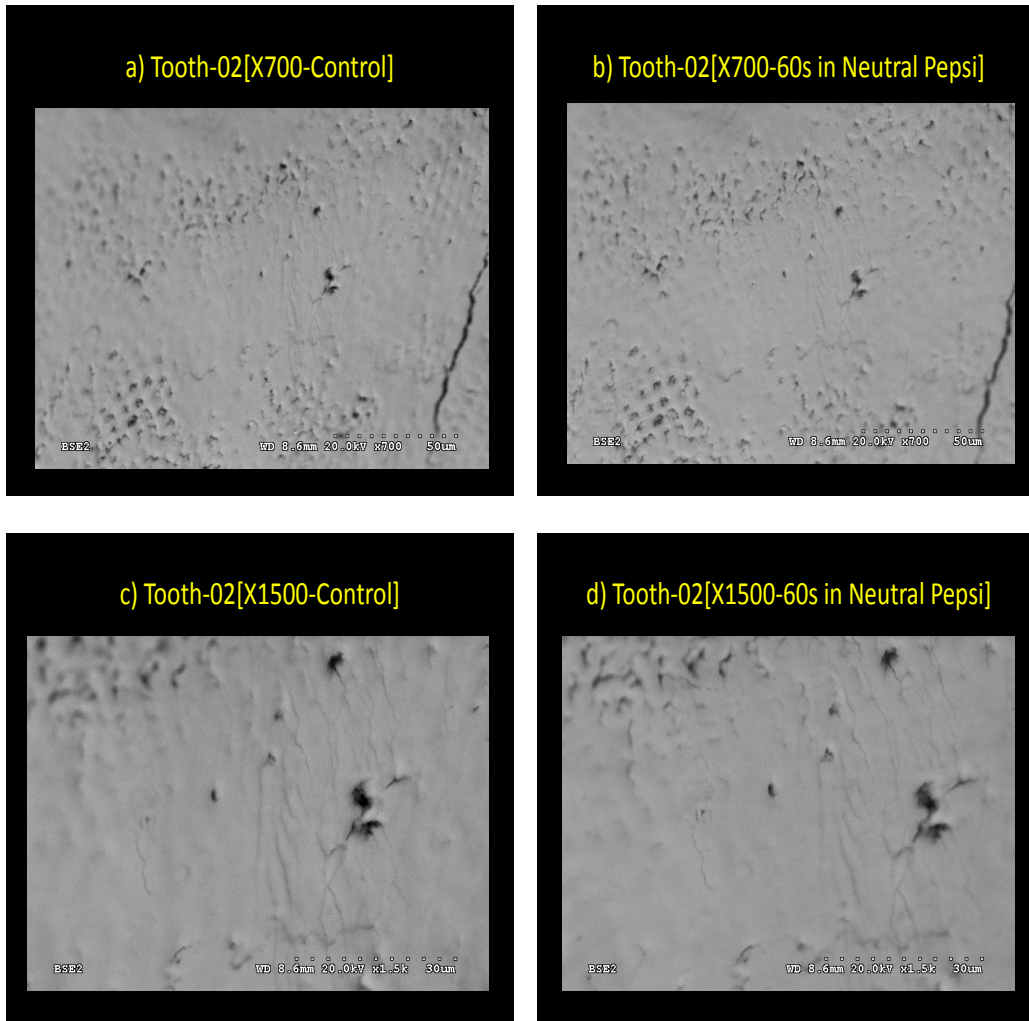


Figure 3-22(Sample02): *Tooth & Neutral Pepsi.*

Areas show minimal surface changes from exposure of teeth to neutral Pepsi when comparing **figures 3-22 (b, d)** to controls in **Figures 3-22 (a, c)**. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented in **Figure 3-5**.

3.5) Discussion

3.5.1 pH, ACIDITY AND BUFFERING: Pepsi-Cola was selected because it is reputedly the most popular brand of Cola consumed in Montreal, Quebec. Both forms of Pepsi-cola (fresh and flat) show a progressive increased requirement of base to neutral pH [pH 7]. In addition to pH value and buffering capacity of pop-colas, other chemical factors such as type of acids, and calcium, phosphate, fluoride content of the drinks and their degree of saturation with respect to teeth minerals are important to explain erosion (20). The pH, buffering capacity and acid reserve of the cola produce the erosive effect on teeth. Although calcium, phosphorous and fluoride are all important in affecting erosion, without an acid environment, the stated elements (Ca, P &F) may not allow erosion. **Figure 3-1** shows the pH of the selected drinks (Aquafina water, and fresh/flat/neutral Pepsi). Both fresh and flat Pepsi drinks register pH values well below the critical pH 5.5, at which tooth decalcification occurs [**Figure 3-1**]. Among the tested drinks, fresh and flat Pepsi with no significant difference, are the most acidic [pH2.60 and pH2.53 respectively] while Aquafina water (Control) is the least [pH5.43]. From the results presented in **Figure 3-2**, fresh Pepsi has the largest buffering capacities for a pH change up to neutralization [pH 7.0]. These results are noteworthy as the low pH values reflect that the both forms of Pepsi, even fresh or flat, are extremely acidic and consequently could all contribute to decalcification of pure hydroxyapatite crystals and human teeth [**See Figures 3-7 to 3-22**]. It is also important that an average of 6.21 mL of base is required to bring the pH of fresh and flat Pepsi back to the neutral level of pH 7.0 [**Figure 3-2**]. This indicates that not only is Pepsi highly acidic when it is first exposed to calcium hydroxyapatite from teeth, but also there is no significant difference between the acidity of fresh and flat Pepsi; they both require large amounts of base to be neutralized *in vitro*. The notion that dissolved CO₂ changes the buffering of cola significantly is substantiated in Fig 3-2.



The hydrogen ion in fresh (gassy) Pepsi becomes active and needs more alkali to reach neutral. Once the Pepsi goes flat, the reaction reverses, and the cola loses CO₂, creates water, and the flat Pepsi needs less alkali to reach neutral pH.

3.5.2 IMMERSION OF TEETH AND NP-GHA IN WATER, AND FRESH/FLAT/NEUTRAL PEPSI *IN VITRO*:

Whole saliva as a complex buffering solution may provide a definite protective buffering and neutralizing effect on acid dietary drinks (38-40). But with acid drinks, including pop-colas, this buffering/neutralizing effect may be overwhelmed (41). The dental and salivary calcium salts absorb some of the phosphorous from the colas; also acidity from colas vary as does buffering and reflex stimuli *in vivo* may vary in intensity of reaction. In this experiment the effect of acid content of fresh/flat Pepsi tested on fresh pristine teeth and granular hydroxyapatite *in vitro*, and the results compared with the effect of water and neutral Pepsi as control all in the absence of saliva. Unlike enamel surfaces in human teeth which is protected by many components in saliva and proteins (30), pure biosynthetic hydroxyapatite has no protection. There is significant decalcification ($p < 0.05$) effect by both fresh and flat Pepsi on pure hydroxyapatite crystals and fresh teeth as the result of acid content of Pepsi; but NP-GHA crystals are more vulnerable than teeth due to lack of saliva, surface pellicles, and proteins protection. Immersion of biosynthetic crystals in Aquafina water, fresh/flat/neutral Pepsi for 60s show rigorous decalcification compared to that with fresh extracted teeth that result in significantly ($p < 0.05$) less decalcification.

In vivo, some phosphates may derive from stimulated saliva, and some from the teeth or other intra-oral phosphate sources [gingivo-crevicular-fluid, and calculus (as tricalcium phosphate, octa-calcium phosphate, dicalcium diphosphate)] (25); *In vitro*, increased phosphorus content compare to the controls are all derived from NP-GHA crystals and

fresh extracted teeth and not from intra-oral sources. The evidence shows higher loss of phosphorous in NP-GHA crystals than in teeth samples. Differences in size, structural and chemical content of all samples provide no certainty for calcium/phosphorus ratios.

3.5.3 SCANNING ELECTRON MICROSCOPY:

Recent increases of consumption of pop-drinks (42) are reflected in increased reporting of dental erosion (43;44). Dental ravages from cola drinks have a high prevalence in children and young adults (45;46). Dental Salivary pellicle is a protein-based layer which forms on surfaces of teeth over time (20). Sixty second exposure of human teeth to acid content of pop-colas will disrupt surface protein aggregation and result in removal of protective layer. The efficacy of salivary protective ingredients and surface alteration can be detected qualitatively by scanning electron microscopy (25). Data reported here shows decalcification occurs at a microscopic level with severe damage to NP-GHA when compared with those in human teeth. NP- GHA lacks pellicles and other protective layers, and accordingly in NP-GHA specimens show deep chemical dissolution of all exposed superficial layers [Figure3-7 to 3-14]. Surface etching and soft layer removal by acid is also observable and marked in teeth but less compare to that in NP-GHA [Figures 3-14 to 3-22]. These SEM photographs are consistent, reinforce and are in concordance with the chemical analysis presented above.

3.6) Concluding Remarks

[i] Fresh and Flat Pepsi-Cola tested are shown to be highly acidic and can potentially decalcify tooth material.

[ii] This study provides evidence that there is no significant difference in the acidity of fresh and flat Pepsi, and both leach calcium out of NP-GHA and teeth, after rinsing with the drink. The cola test-drinks, after NP-GHA immersion, reveal a

significant increase [$p < 0.05$] of calcium, found in all the liquids tested compared to the control (Aquafina Water). Similar, but less decalcification expressed as mg/L, from the source and for test-drinks after immersion with a tooth, was observed. Very small amounts of calcium leach out from teeth in water and neutralized Pepsi; the amounts are not significantly different between these two. Higher phosphorus concentrations were detected in the liquids after NP-GHA immersion except for Aquafina water. Increase in Phosphorus concentration is due to leaching of phosphorus ions out of NP-GHA crystals. Also higher phosphorus concentration detected after immersion of teeth into drinks except for Aquafina water and neutral Pepsi. Increase in phosphorus concentration is due to leaching of phosphorus ions out of teeth crystals in the more acid medium. Comparison of ICP-OES measures between the two samples (NP-GHA and teeth) show more de-calcification in NP-GHA than in teeth.

[iii] The SEM experiment reveals that after 60s cola exposure *in vitro*, acid Pepsi ignores the smear layer and other organic components, erodes dental hard tissues, and modified surface crenellations develop.

[iv] This study consistently demonstrates, and provides clear chemical and SEM visual evidence, that NP-GHA and enamel morphology is altered by acid decalcification from short exposure to Pepsi-Cola.

Chapter 04

Pop-Cola Acids and Tooth Erosion

An in vitro, in vivo, electron-microscopic and clinical report

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4.1) Abstract

Introduction: Manufactured Colas are consumed universally as soft drinks. Evidence about the acid contents of Cola-beverages and its effects on teeth is rare.

Aim: To assess: (i) cola acidity and buffering capacity *in vitro*, (ii) tooth erosion after swishing with colas *in vivo* (iii) *scanning electron microscopic effects* on teeth of colas, and tooth-brush abrasion, and (iv) report *a clinical case* of erosion from cola consumption.

Materials and Methods: (i) We measured six commercially available pop ‘Cola-beverages’, pH and buffering capacities using a pH-Mettler Automatic Titrator, with weak solution of Sodium Hydroxide. (ii) Two cohorts, one *with teeth*, the second *without teeth* rinsed with aliquots of Cola for 60 seconds.

Swished cola samples tested for calcium and phosphorus contents using standardized chemical analytical methods. (iii) Enamel, dentine and the enamel-cemental junction from unerupted extracted wisdom teeth were examined with a scanning electron microscope after exposure to colas, and tested for tooth-brush abrasion and (iv) a clinical case of pop-cola erosion presentation, are all described.

Results: Comparisons among pop-colas tested *in vitro* reveal high acidity with very low pH. Buffering capacities in milliliters of 0.5M NaOH needed to increase one pH unit, to pH 5.5 and pH 7 are reported. Rinsing *in vivo* with pop-cola causes leaching of calcium from teeth; SEM shows dental erosion, and pop-cola consumption induces advanced dental erosion and facilitates abrasion.

Conclusions: (i) Pop-Cola acid activity is below the critical pH 5.5 for tooth dissolution, with high buffering capacities countering neutralization effects of saliva; (ii) calcium is leached out of teeth after rinsing with pop-colas; (iii) SEM evidence explains why chronic exposure

to acid pop-colas causes dental damages; and (iv) a *clinical case* of pop-cola erosion confirms this.

Key Words: Acid, Attrition, Abrasion, Beverages, Buffering, Calcium, Cola, Coca-Cola, Diet-Coke, Diet-Pepsi, Diet-Selection, Erosion, Frangibles, Pepsi, Selection-Cola, Teeth.

Supported by a grant [Number 217 000] from Office of the Vice Principal Research McGill University.

Acidité du cola et érosion dentaire

Rapport d'étude in vitro, in vivo,

par microscopie électronique et clinique

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4.1(A) : Résumé

Introduction : Il se consomme des boissons gazeuses de type cola partout dans le monde. Les éléments de preuve concernant la teneur en acide des boissons de type cola et ses effets sur les dents sont rares. **Objectif :** (i) Mesurer l'acidité et le pouvoir tampon du cola *in vitro*, (ii) mesurer l'érosion dentaire à la suite d'un rinçage *in vivo* avec des colas, (iii) mesurer les *effets* du cola sur les dents *au moyen d'un microscope électronique à balayage*, ainsi que l'abrasion découlant du brossage, et (iv) présenter un *cas clinique* d'érosion issue de la consommation de cola. **Matériel et méthodes :** (i) Nous avons mesuré le pH et les pouvoirs tampons de six boissons gazeuses commerciales de type cola au moyen d'un pH-mètre et d'un titrimètre automatique de Mettler, avec une solution faible d'hydroxyde de sodium. (ii) Deux groupes, l'un dont les membres avaient des dents et l'autre non, se sont soumis à un rinçage de 60 secondes avec une aliquote de cola. La teneur en calcium et en phosphore des échantillons se rapportant au rinçage avec du cola a été mesurée au moyen de méthodes normalisées de chimie analytique. (iii) L'émail, la dentine et la jonction émail-cément des troisièmes molaires extraites n'ayant pas fait leur éruption ont été examinés au microscope électronique à balayage après l'exposition au cola, et

ont été soumis à un test d'abrasion par brosse à dents, et (iv) un cas clinique d'érosion attribuable au cola est présenté. **Résultats** : Les comparaisons entre les colas testés *in vitro* révèlent une acidité élevée avec un pH très bas. Les pouvoirs tampons en millilitres de NaOH 0.5 M devaient augmenter d'une unité de pH, et les mesures de pH 5.5 et pH 7 sont rapportées. Le rinçage *in vivo* avec du cola entraîne la décalcification des dents. En effet, les examens réalisés au microscope électronique à balayage montrent une érosion dentaire. Par conséquent, la consommation de colas provoque une érosion dentaire avancée et contribue à l'abrasion. **Conclusions** : (i) L'activité acidifiante du cola se situe sous le seuil critique de pH 5.5 pour la dissolution dentaire, et présente un pouvoir tampon élevé qui annule les effets neutralisants de la salive; (ii) le calcium est éliminé des dents après le rinçage avec les colas; (iii) les analyses réalisées au microscope électronique à balayage expliquent pourquoi l'exposition chronique à l'acidité des colas entraîne une fragilité dentaire ; et (iv) un *cas clinique* d'érosion due au cola vient confirmer les résultats obtenus.

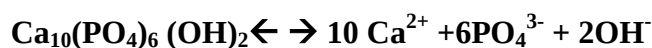
Mots clés : Acide, Attrition, Abrasion, Boissons, Tamponnage, Calcium, Cola, Coca-Cola, Coke diète, Pepsi diète, Sélection diète, Érosion, Fragile, Pepsi, Sélection cola, Dents.

Cette recherche bénéficie d'une subvention [numéro 217 000] du bureau du vice-principal, Recherche, Université McGill.

4.2) Introduction

Acidic foods are consumed worldwide (24;47), but often are viewed to be innocent as to their effects on the oral health. Carbonated beverages have many different confounding factors (fruit acids, derivatives, flavorants, alcohol and preservatives). Among all carbonated beverages consumed globally, acidic colas are universally most popular and frequently consumed, and consequently colas as a generic group were selected for investigation. Chronic exposure from acidic food and beverages can effect natural teeth and frequently instigates the development of dental damages [attrition, erosion, abrasion and decay] (43). Of critical importance is that acid is not only derived directly from colas, but also after drinking the cola, biofilm bacteria metabolizing with fermenting sugars in the drinks creates more acid. (48). Research demonstrates human saliva acts as a neutralizing and/or buffering solution on imbibed acid beverages (31;48). Intra-oral pH [pH 6.8] decreases measureably after drinking an acidulated drink to below pH5 within 2 to 3 minutes (31). Additionally, oral acidogenic bacterial action on fermentable carbohydrates [monosaccharides like glucose and fructose; disaccharides like maltose and sucrose] is a causal factor that aggravates the pH reduction to below pH4. Intra-oral pH takes about 25 minutes to change the acid environment, as further stimulated-saliva neutralises any residual acid (31). The critical pH, at which hydroxyapatite dissolves, is pH5.5 and because teeth are composed of *calcium-deficient carbonated hydroxyapatite*, they are vulnerable to decalcification in acid media (22). Depending on incumbent acidity of the surrounding saliva, tooth material in saliva as a saturated

solution of calcium and phosphate, will show dissolution or remineralisation. This is illustrated by the formula:



Should the pH fall [increased H⁺ concentration and accordingly acidity], the 6PO₄³⁻ ions convert to H₂PO₄²⁻ or H₂PO₄⁻, and the OH⁻ ions are neutralized to water, driving the reaction to the right i.e. dissolution of calcium. If the H⁺ concentration is lessened [i.e. pH rises and acidity reduces] the reaction is driven to the left producing re-mineralization (22;31). Continual exposure to acid drinks changes the intra-oral pH to below the critical pH [pH5.5], when chemical dissolution of calcium from dental hydroxyapatite occurs. This sustained low pH, <pH5.5, permits mordant development of dental damages starting and deriving from decalcification. Calcium, phosphate and to a lesser extent fluoride should be considered important agents in conjunction with pH levels and buffering in the erosive effects from beverages (22).

Evidence reveals from research that considers the method of drinking, the frequency of intake, timing of consumption as well as the type and quantity of beverages, influence the development of dental damages (43). There is modest data presented by the makers of pop-Cola beverages detailing the acidity level, and the formulae and contents of said products are closely guarded industrial secret. With the knowledge that acid drinks require a lot of alkaline salivary flow to neutralize dietary acid and the critical pH level for hydroxyapatite, the following criteria must be determined: 1) The pH of pop-Cola drinks, and 2) the strength of their acids, specifically, their buffering capacities as to how much alkali is needed to change their pH.

Multiple methods are used to compute the ultra-microscopic effects of beverages on teeth. These include: surface hardness measurements; surface profilometry, iodide permeability tests, chemical analysis of dissolved minerals, micro-radiography, confocal scanning microscopy, quantitative light induced fluorescence, atomic force microscopy, element analysis of solid samples, nano-indentation, ultrasonic measurements, and scanning electron microscopy [SEM] with environmental SEM [ESEM] (46). With ESEM minimal sample preparation is required; it is economical and minimizes risks for artefacts. Also ESEM and SEM allow for progressive assessment of the identical test-tooth material without carbon or metal coating of native surfaces(46). Recently [2010] Variable Pressure Scanning Electron Microscope [VP-SEM] has refined this technique further (37).

4.3) Aim

The aim of this study is threefold:

- [i]** To determine pH and buffering capacities of six ‘pop-cola-beverages’ (initial rise of one pH unit, to the critical pH, and to neutral pH).
- [ii]** Measure calcium and phosphorous content of cola after rinsing from mouths with (dentate), and without teeth (edentulous).
- [iii]** Assess scanning electron microscopic [VP-SEM] erosive effects on native dental hard tissues after exposure to pop-colas.
- [iv]** Describe a clinical case of pop-cola erosion.

4.4) Methodology

4.4.1 PH AND BUFFERING CAPACITY: The following six pop-cola were analyzed:

Pepsi, Diet Pepsi, Coca-Cola, Diet Coca-Cola, Selection Cola and diet Selection Cola. Standard aliquots of Cola-liquid were tested for pH and buffering capacity. Using an automated METTLER DL25 Titrator [AT], the acidity as pH and buffering capacity were measured. (32). Then with a 0.5 M NaOH solution, buffering capacities were assessed, and titrated to raise the measured cola pH one pH unit, then to pH 5.5, and to pH 7. Two fresh 60mls aliquots were then collected directly from each of 12 cans, and placed in separate polystyrene Falcon test-tubes for measurements, after which titration measures were repeated for each sample from different cans in the same batch. Thus, for each of the 6 Colas, 24 titration measures were done.

4.4.1(A) AUTOMATIC TITRATOR [AT]:

In order to determine and measure the pH and buffering capacities of the various pop-cola liquids, the AT was utilized according to described methods by Larsen et al 1998 method (33), and measured initial pH's [**figure 4-1**] and buffering capacities at the three different stated levels: (i) pH change of one unit from the incipient pH [orange bars **figure 4-2**], (ii) pH change up to the critical level pH 5.5 [red bars **figure 4-2**], and (iii) pH change up to the neutral level pH 7.0 [green bars **figure 4-2**] (49). All measures were assessed at room temperature of 23°C. Ensuring measurement accuracy and to establish base line controls, the AT was calibrated each time before use, at pH 2, 4, 7 and 10, using standard buffer solutions for all levels. A 60 mL volume of the tested sample was then titrated using a 0.5M NaOH

solution. Measures were repeated 24 times; the means of these are reported. **See Appendix I.**

4.4.1(B) RESULTS AND STATISTICS:

In this chapter, separate comparisons were done between the two groups (with teeth vs. without-teeth) for each type of drink. Because the contrasts were pre-specified, there was no need for an ANOVA analysis. Instead we performed independent samples *t*-Tests for each drink. Bootstrap two sample *t*- Test used to identify the difference between two groups (with teeth & without teeth) for each drink, separately for Calcium and phosphorus. P-values were adjusted for multiple testing using the Hochberg’s method (36).

See **figures below**. Details of statistical analyses and all p-values stated in the text reported in **Appendix IV (Table 03)**.

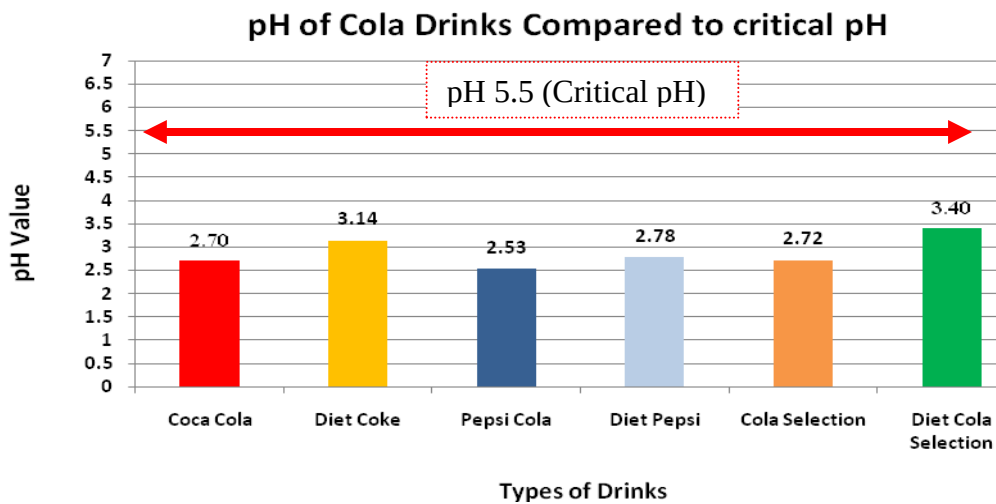


FIGURE 4-1: pH COMPARISON AMONG THE DRINKS: The 6 drinks analyzed showed the pH levels are all notably below the critical pH [pH5.5] of calcium-hydroxyapatite [$p<0.05$]. The diet-colas are not necessarily more acidic. Regular

Pepsi Cola has the lowest pH [pH 2.53], while Diet Selection Cola is the highest pH [pH 3.40].

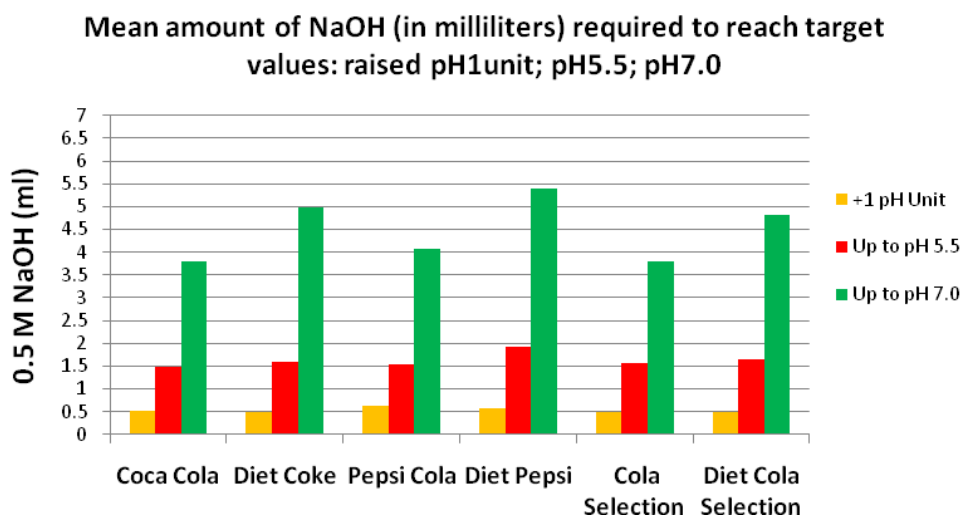


FIGURE 4-2: BUFFERING CAPACITIES OF POP COLA DRINKS FOR A SINGLE UNIT PH CHANGE (ORANGE), TO CRITICAL PH5.5(RED) AND PH7 (GREEN) : The orange bars show Pepsi-Cola having the highest buffering capacity while Diet-Coke and Selection Cola share the lowest buffering capacity. The red bars indicate buffering capacities for change from the drink's initial pH up to the critical pH 5.5. Here Diet Pepsi is found to have the highest buffering capacity [1.9ml of 0.5 M NaOH] while Coca Cola has the lowest buffering capacity [1.5ml of 0.5M NaOH]. The green bars display the buffering capacities for a pH change from the drink's initial pH up to neutral [pH 7.0]. Diet Pepsi has the highest buffering capacity [5.38 ml 0.5M NaOH], regular Selection Cola [3.78 ml of 0.5M NaOH] has the lowest buffering capacity, and Coca Cola [3.79 ml of 0.5M NaOH] is very similar and not

notably different from Selection Cola. All the cola-drinks absorb the alkali, but vary in the amount of alkali to reach pH7.

4.4.2 RINSING POP-COLAS WITH AND WITHOUT TEETH:

The six different pop-colas previously mentioned [Coca-Cola, Pepsi-Cola, Selection-Cola, Diet-Coke, Diet-Pepsi, and Diet-Selection-Cola] were tested. Two volunteer cohorts, one fully dentate [mean age 22, M: F 4:2, n = 6] the second edentulous [mean age 52, M: F 3:3, n = 6], were used to swish with 20ml aliquots of de-ionized Aquafina water as baseline, and subsequently a 20ml aliquots cola for 60 seconds for each volunteer. Each cola was sampled and analyzed twice, providing 12 analyses for swishes, using standardized analytical chemistry methods (34). The colas from both source and post-swish expectorates were analyzed for calcium, and phosphorous contents using Inductively Coupled Plasma with Optical Emission Spectroscopy [ICP-OES] (34), and an Ion Chromatography [IC] was used to assess concentrations of anions (Fluoride) present in the six colas direct from their cans. **[See Appendix II & III]** For our analysis, concentrations of calcium and the major ions of interest as phosphate and fluoride are reported here (35). To maintain neutrality as to eliminate inter- and intra-operator bias, data was analyzed ‘blind’ by third party technicians unaware of the source of procured samples.

4.4.2(A) RESULTS AND STATISTICS: Analysis shows consistent and significant [p<0.05] increases in calcium leached from dentate subjects after swishing with all the pop-colas tested. After comparing the ICP-OES and IC measures, no crucial differences were found in respect to results obtained for phosphorous. In the tests, the variable amounts of phosphorous found in the various pop-colas were also

reflected in the swish analysis of phosphorous present after rinsing with these pop-colas. Hard tissue erosion as a result of chemical dissolution of calcium, may differ between colas, but is produced by all the colas tested. Hard tissue erosive action is present in all tests with pop-colas after post-swishing exposure and *calcium content in the swish expectorates with teeth all showed significant [$p<0.05$] increases in dissolved calcium when compared to swishing with water, or swishing without teeth* [See Figure 4-3].

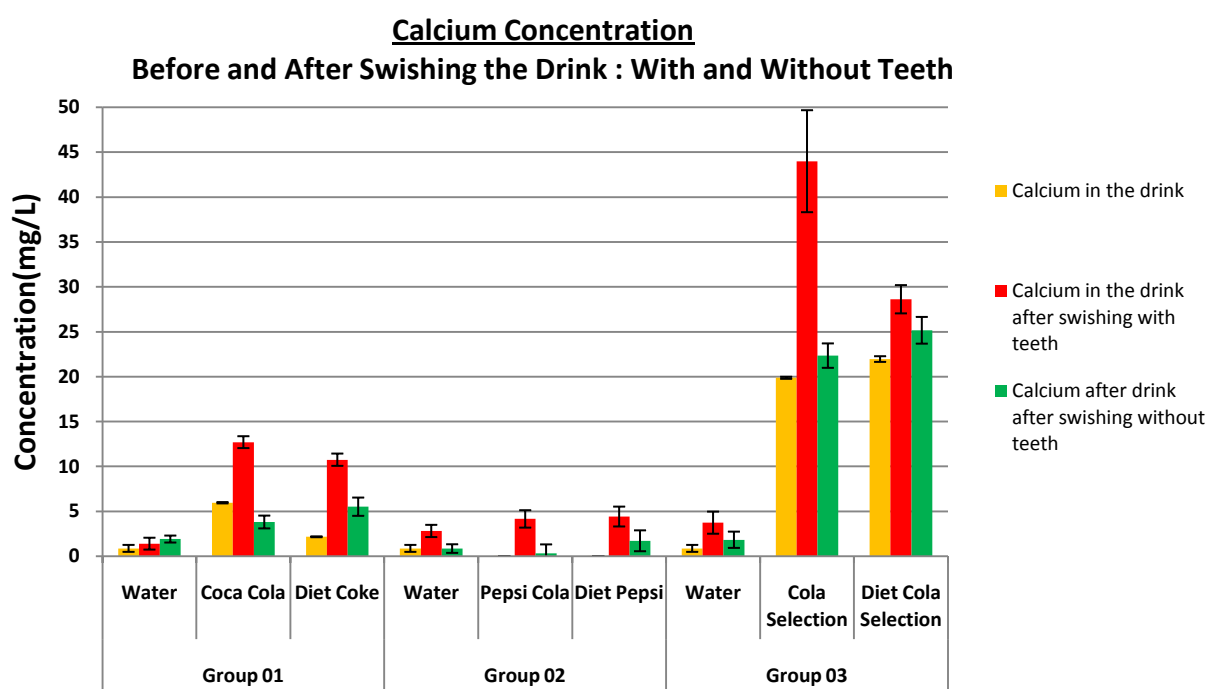


FIGURE 4-3 CALCIUM MEASURES OF SWISHES: Using the same water (Aquafina), the initial swishes were done for all groups as controls. After which test standard-cola swishes were taken; 2 hours later diet equivalent swishes were gathered. Calcium measures were taken direct from source [bottled water or can] and the swished expectorates. Measures were achieved using ICP-OES. The Ca^{2+} content of control (water) remains constant for each group; but the Ca^{2+} content of swished

water probes obtained from 3 separate cohorts (six volunteers for each group) vary slightly. Calcium expressed as mg/L from the source and for test-colas after *swishing with and without* teeth. When comparing the swishes of cola from subjects *with-teeth* to those swishes of colas from subjects *without-teeth*, a significant increase [$p<0.05$] of calcium is found in all the colas tested. The calcium content in the water controls is negligible.

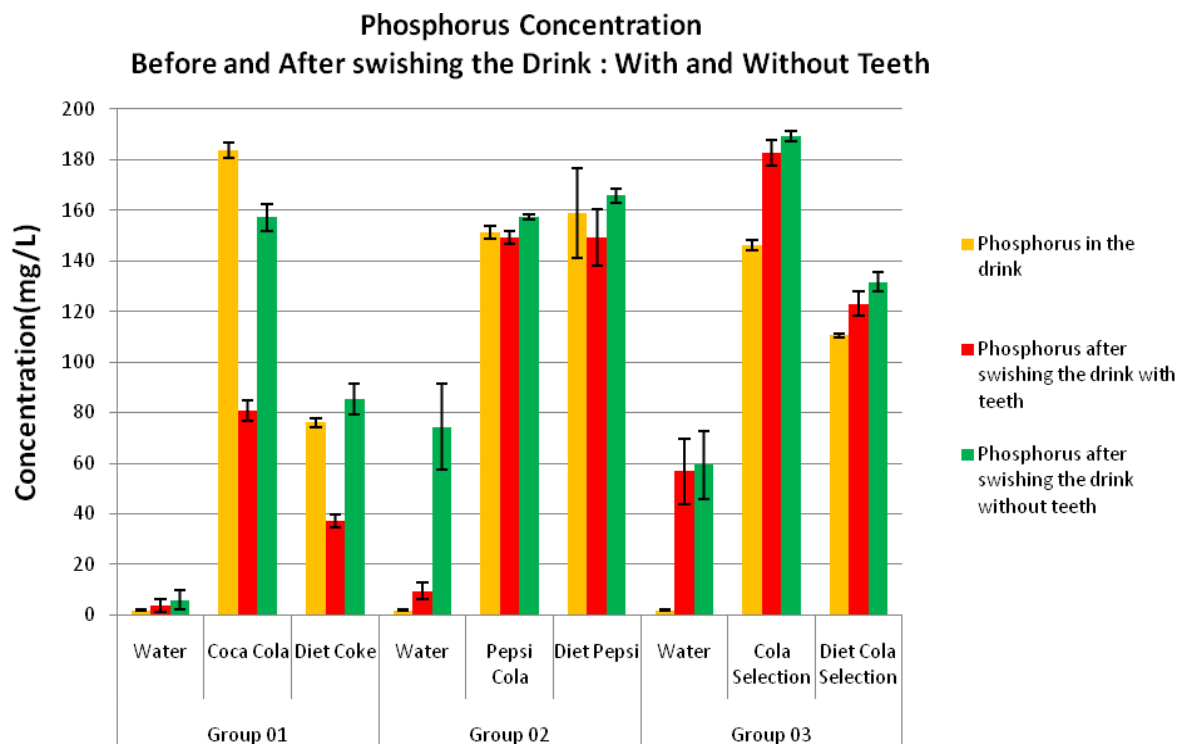


FIGURE 4-4 PHOSPHOROUS CONCENTRATIONS: Expressed as mg/L from the source [bottled water or can] and swished test-drinks. Once again, Aquafina water used as a control for all groups. Measures were gathered using ICP-OES. The phosphorus content of control (water) remains constant for each group; but the phosphorus content of swished water probes obtained from 3 separate cohorts (six volunteers for

each group) vary. Phosphoric acid is an added main constituent in all the colas tested. This is the main source of Phosphorous in the swishes. What is revealed is that there is wide variation of phosphorous concentrations when swishes with cola from subjects *with teeth* are compared to swishes of cola from subjects *without teeth* (For more detail please refer to discussion (4.5.2).

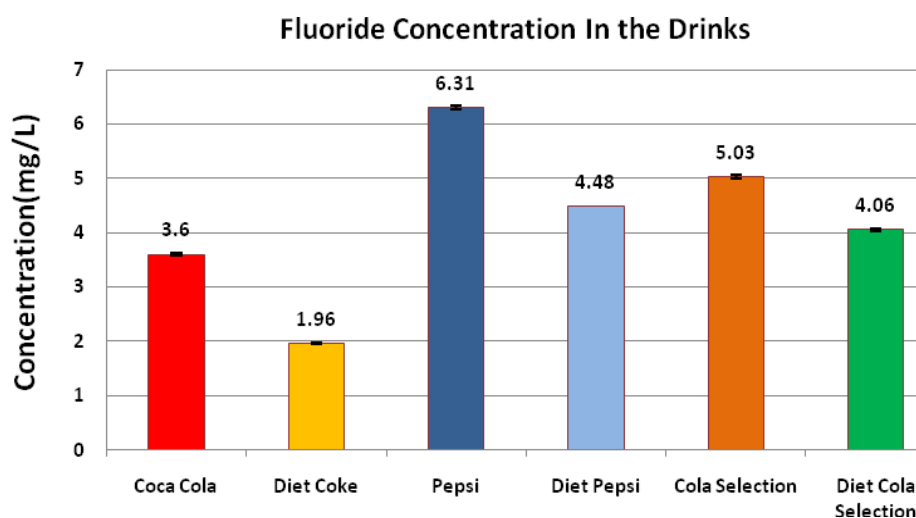


FIGURE 4-5 FLUORIDE CONCENTRATION IN POP-COLAS WITH SAMPLES [N=24]

DIRECT FROM THE CANS: The above graph indicates the fluoride concentration [mg/L or ppm] in the six Cola drinks tested. Pepsi has the highest concentration [6.31 ppm] while Diet Coke has the lowest concentration [1.96 ppm].

4.4.3 SCANNING ELECTRON MICROSCOPY (SEM):

For this procedure, healthy teeth condemned for extraction [orthodontics or impactions] were used. Immediately after extraction, teeth were placed in de-ionized water refrigerated at 4°C for a maximum of two hours. Each cola was tested at three selected sites on the same tooth. This was repeated on three teeth from the same set

of wisdom teeth, which provided fresh native material for assessment. For enamel, flat tooth surfaces were selected; for dentine, samples were obtained by cutting horizontally through the middle of the crown, and the enamel-cemental junction on each tooth used was also tested and examined direct. To section the teeth, a Buehler saw with a diamond cutting blade was used. (50). Following the cutting of the samples, each specimen was rinsed with de-ionized water and then air stream dried. Using scanning electron microscopy with VP-SEM (37), all areas of interest on the teeth were examined. Resolution was at 700X, with accelerating voltage 20-25 kV, to determine and identify fracture patterns and morphology. Immediately after scanning controls, each sample was then immersed in a cola for 30s, and following this, the specimens were immersed for a further 30s [for a total of 60s] and the *same location* was again scanned and recorded. Unadulterated tooth surfaces of the same native precise areas were examined. No sputter, coating, metal or gold plasma enhancement was used. Image analysis of morphology, properties and characteristics [cracks, openings and changes of identifiable shapes] were done with microscopic scales and by micrometer measures using the same microscope magnification of the identical areas tested. After 60 seconds cola exposure the specimens were brushed with a standardized rotary tooth brush for 5 seconds under 60 grams pressure. The specimens were scanned again after brushing.

4.4.3(A) RESULTS:

Ultra-scaled measures [700X] display critical losses of hard tissue from erosion [$p < 0.05$]. Following exposure to colas, there is initial erosion observed as the chemical loss of calcium widens cracks, and causes surface crenellations to develop. After 60s cola exposure, soft surface crenellations develop. Additionally, brushing of teeth presents evidence of tooth-brush abrasion.

See Figures 4-6 to 4-11 next pages:

Coca-Cola (X700)

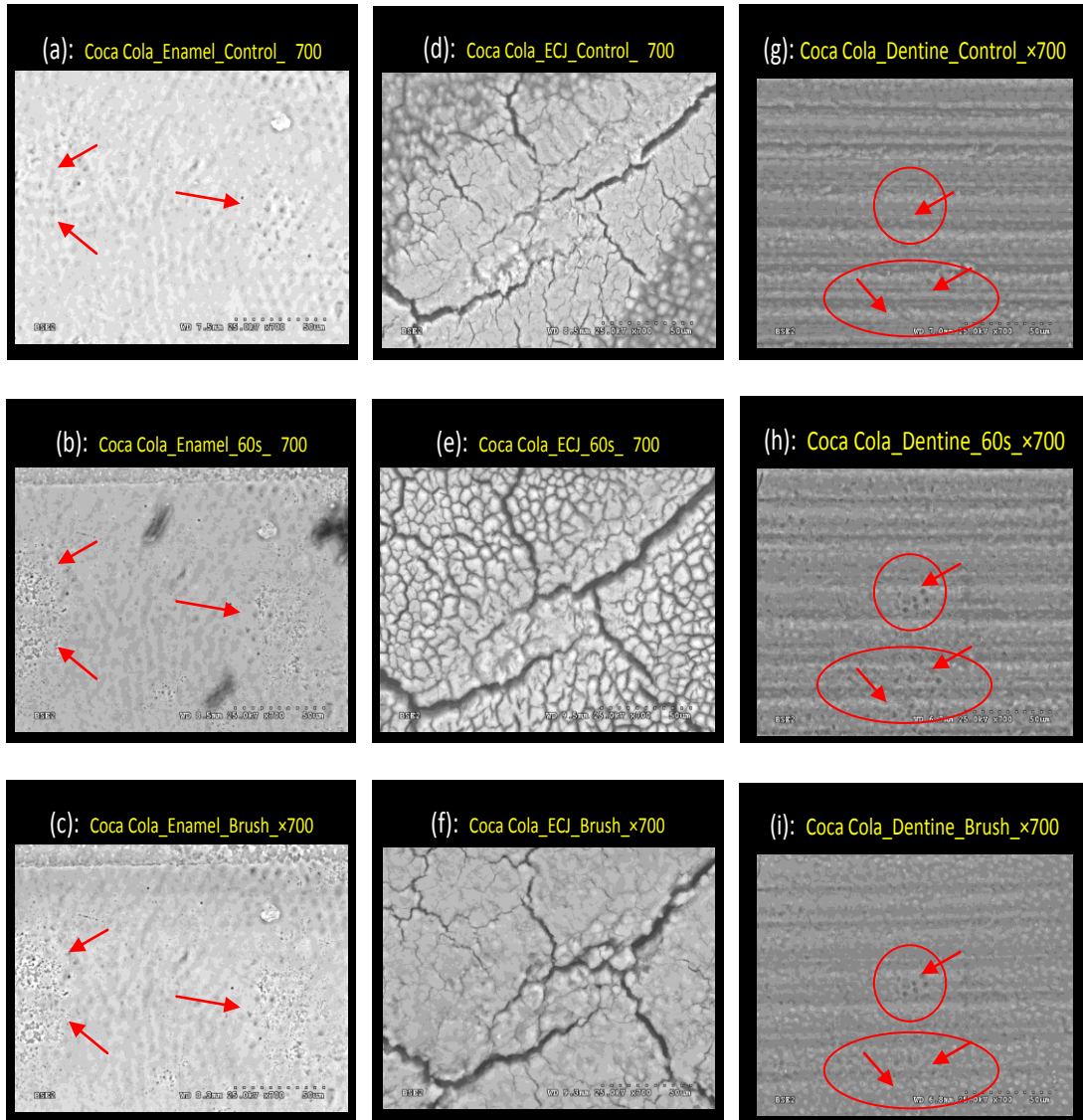


Figure 4-6: Coca Cola ($\times 700$): **Figure 06(a-c):** The red arrows show developing erosive effects on smear layer over the surface of enamel; there is enamel erosion and minor abrasion, as enamel is dense and hard. **Figure 06(d-f)** show developing cracks and crevices over the ECJ surface which becomes aggravated due to loss of calcium. Also abrasion as loss of material after brushing, with the softened surface reflecting loss of detail going down to deeper more calcified layers is seen in **Figure 06(f)** compare to **Figure 06(e)**. Red arrow in **Figure 6 (g)** shows closed dentine tubules which are markedly opened **Figure 6(h)** after exposure to the cola. **Figure 6(i)** shows loss of surface material; removed by abrasion, with some tubules (circled) becoming smaller, while others (red arrow) expose deeper levels of the tubes. These results correlate well with the calcium measured in expectorates from swishes with Coca- cola in **Figure 3**.

Pepsi-Cola (×700)

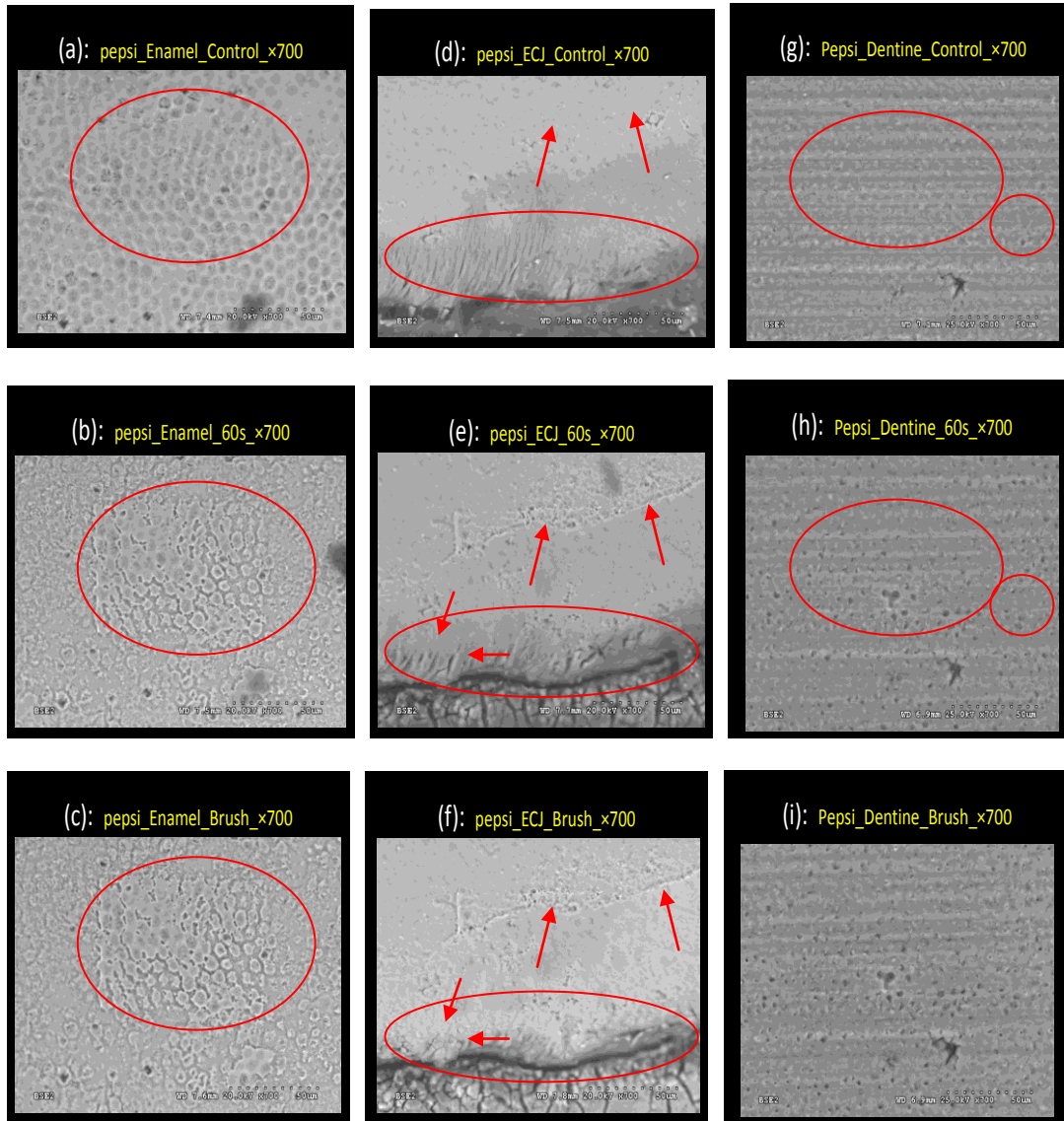


Figure 4-7 (a-i): Red ovoid outlines shows erosion in the same areas for enamel, ECJ and dentine. Red arrows indicate obvious locations of change. There is minimal abrasion in **Figure 7(c)**, as enamel is dense, hard and resistant to the brushing. But red outline in **Figure 7(e)**, shows erosion, and red arrows show loss of surface material from brush abrasion in **Figure 7 (f)**. These SEM results are consistent with the calcium dissolution from swishes in **Figure 3**.

Cola Selection (×700)

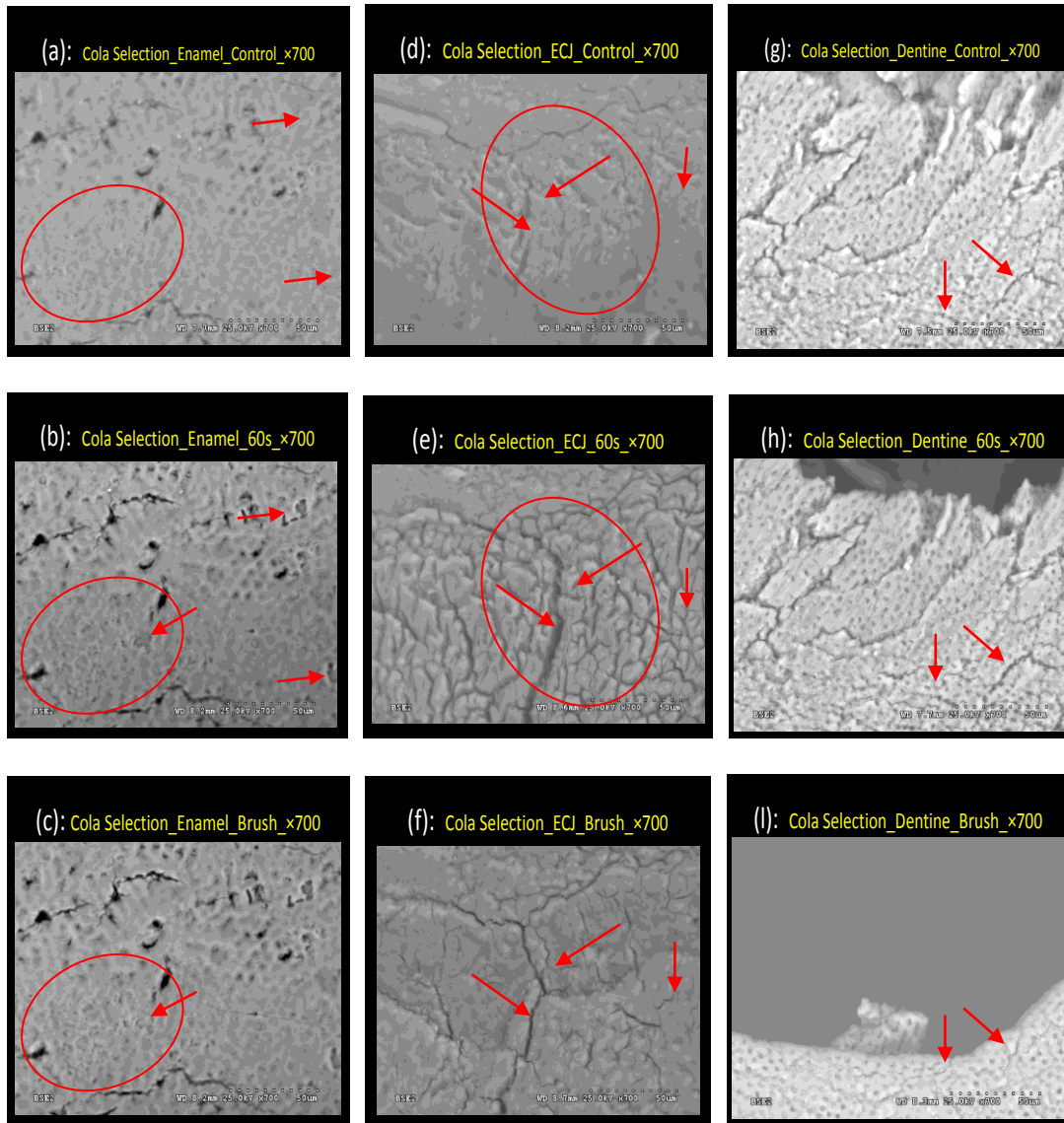


Figure 4-8: Outlined areas clearly show erosion with surface crenellations and profusion of shrinkage- cracks from calcium loss when comparing **figures 8 (a, d, g)** to **Figures (b, e, h)**. Minimal abrasion is present on the enamel, but red arrows from brush abrasion is clearly visible in **Figure 8(f)**; the surface cracks while enlarged from erosion, appear narrower and less numerous, as the soft superficial material is lost to the action of the brush. Comparing **figures 8 (g & h)** show developing mordant effects on dentine. The specimen lost some delicate scaffolding at the top, but retained enough material to demonstrate changes in the dentine. After brushing [**Figure (I)**], an even larger portion disintegrated. These SEM results are also consistent with the calcium dissolution from swishes in **Figure 3**.

Diet Coke (×700)

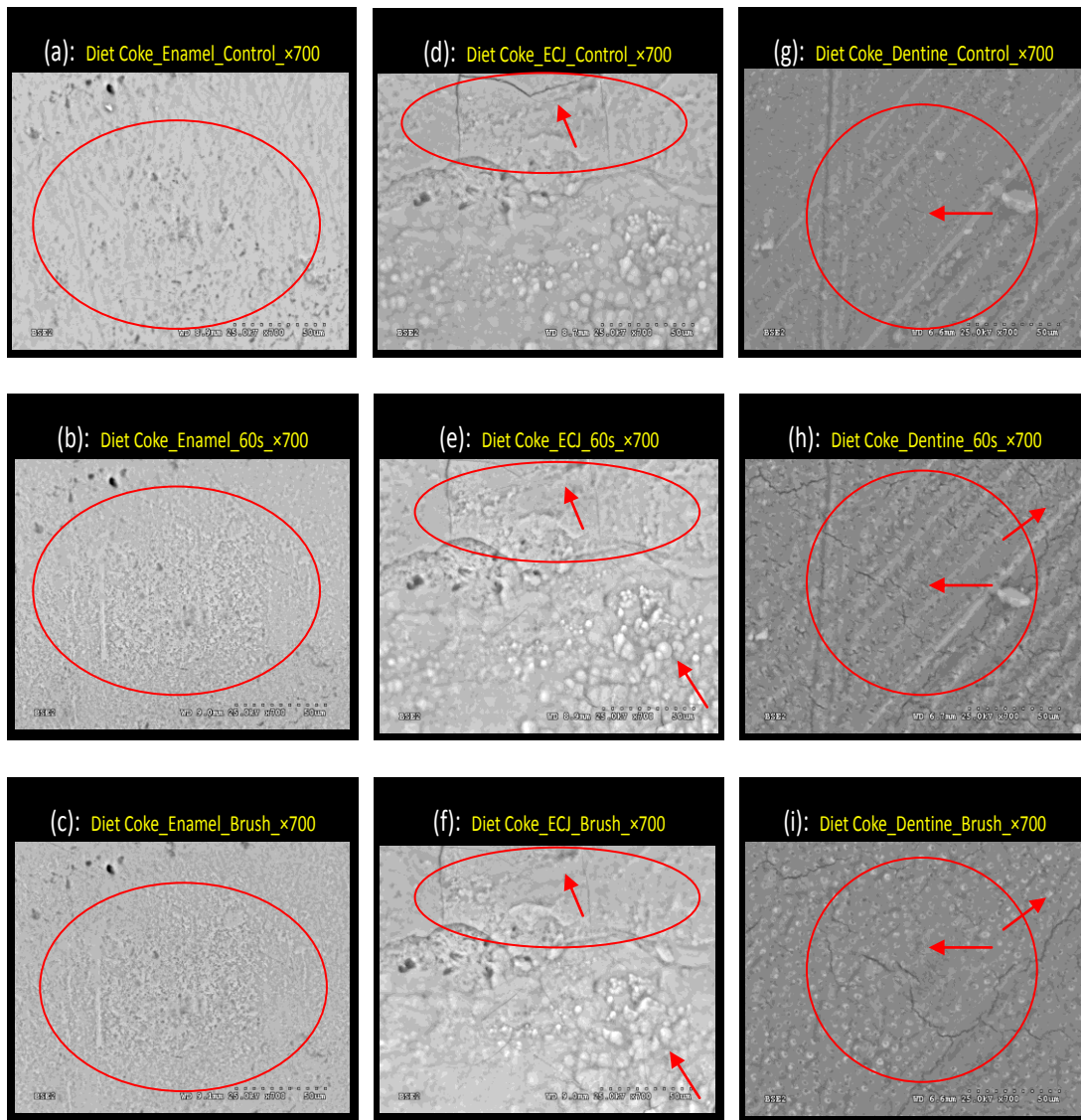


Figure 4-9: Outlined areas show erosion with minor surface changes from calcium loss when comparing **figures 9 (a, d, g)** to **Figures 9 (b, e, h)**. Minimal abrasion is present on the enamel, but red arrows, from brush abrasion, is clearly visible in **Figure 9(i)**. Red arrows show dentine tubules exposed from erosion appear clearer, wider and more open as the soft surface material is lost to brush abrasion. These SEM results are also consistent with the calcium dissolution from swishes in **Figure 3**.

Diet Pepsi (×700)

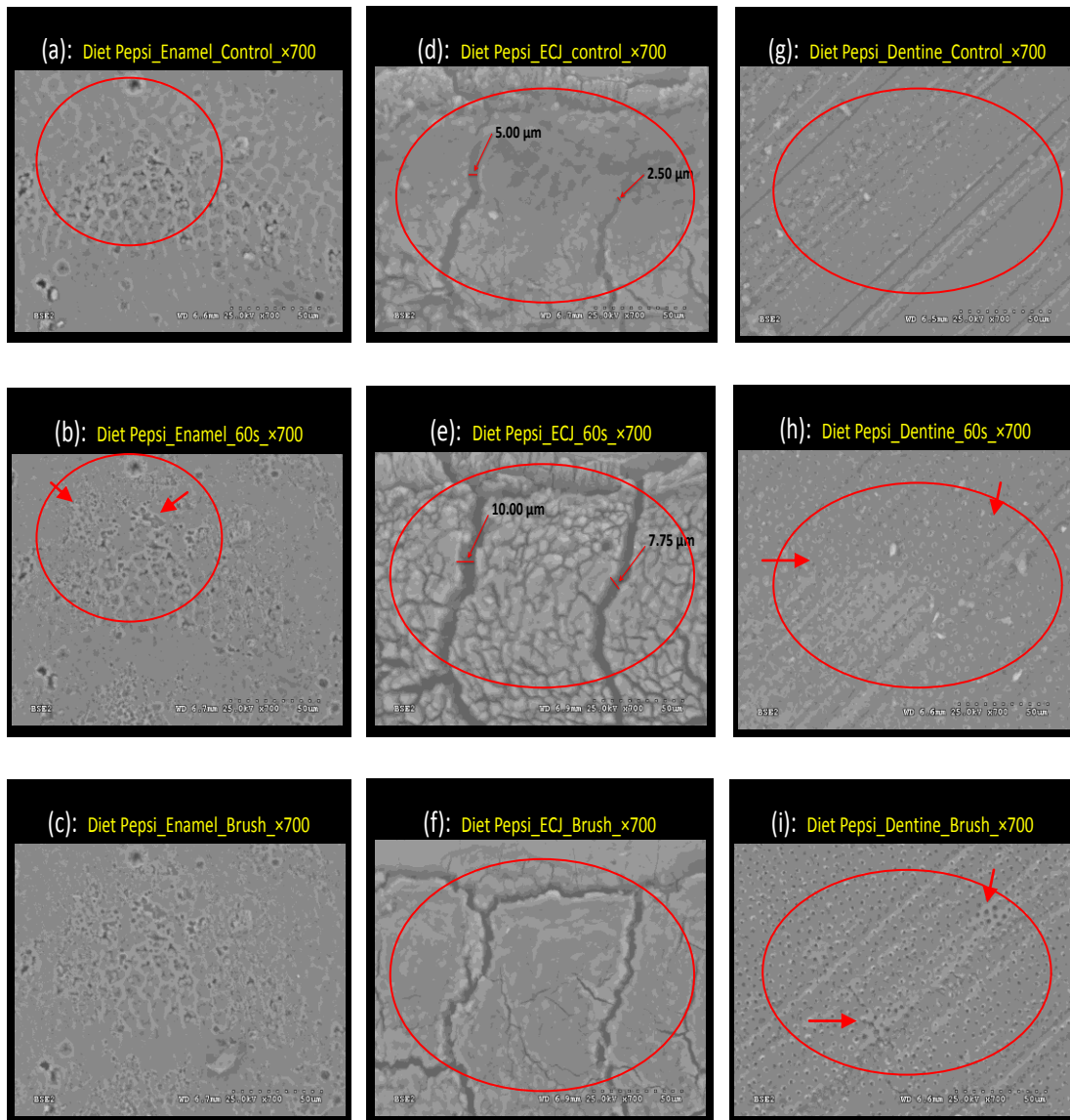


Figure 4-10: Outlined areas clearly show erosion with surface crenellations and profusion of shrinkage-cracks from calcium loss when comparing **figures 10 (a, d, g)** to **Figures 10 (b, e, h)**. Minimal abrasion is present on the enamel, but red arrows from brush abrasion is clearly visible in **Figure 10(f)**; the surface cracks while enlarged from erosion, appear narrower and less numerous, as the soft superficial material is lost to the action of the brush. Red arrows show dentine tubules exposed from erosion appear clearer, wider and more open as the soft surface material is lost to brush abrasion. These SEM results are also consistent with the calcium dissolution from swishes in **Figure 3**.

Diet Cola Selection (×700)

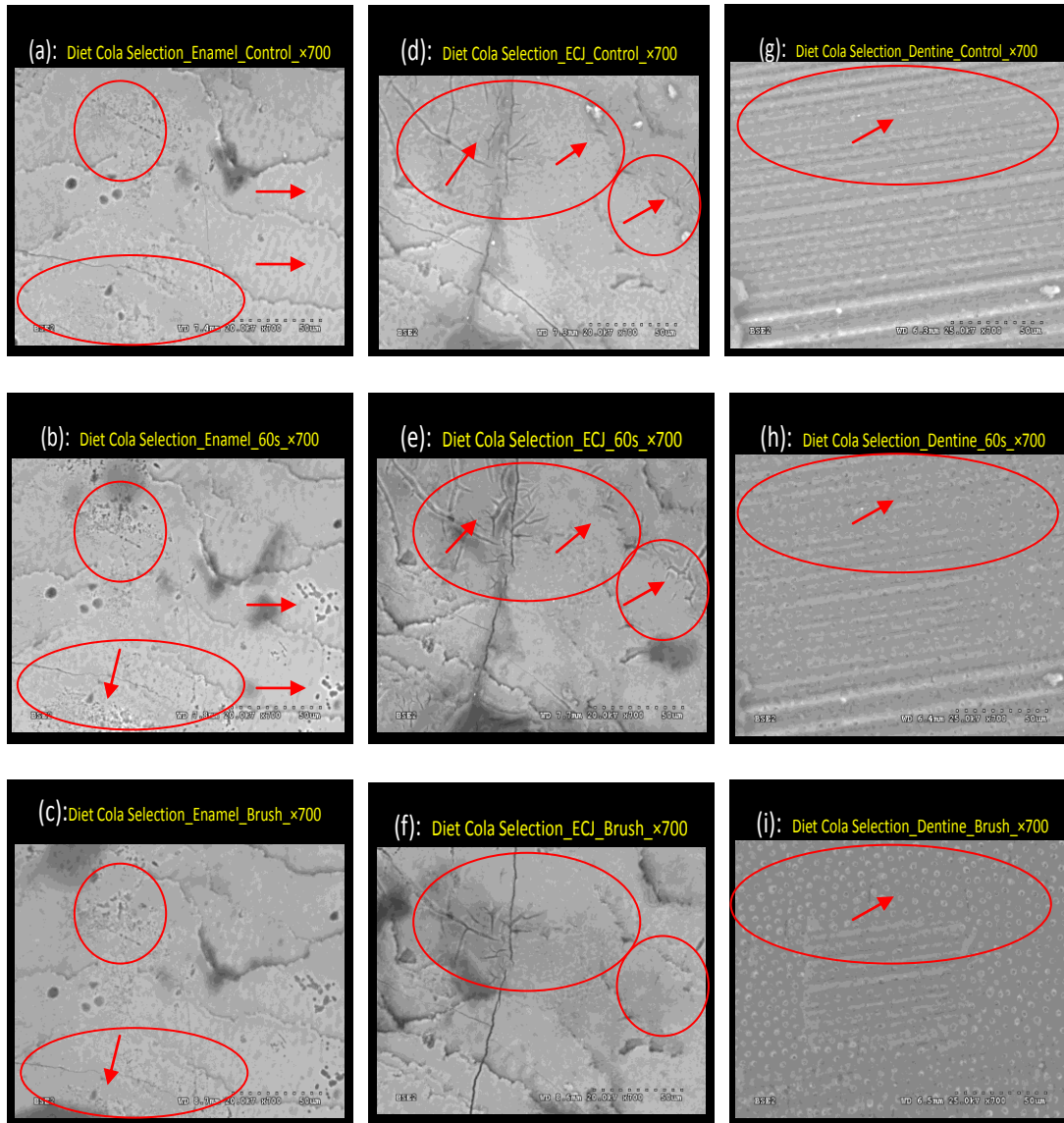


Figure 4-11: Red outlined areas clearly show erosion with surface crenellations and profusion of shrinkage-cracks from calcium loss when comparing **figures 11(a, d, g)** to **Figures 11 (b, e, h)**. Minimal abrasion is present on the enamel, but loss (red arrows) from brush abrasion is clearly visible in **Figure 11(f)**; the surface cracks while enlarged from erosion, appear narrower and less numerous, as the soft superficial material is lost to abrasion. Red arrows in **Figure 11 (g-i)** show dentine tubules exposed from erosion appear clearer, wider and more open as the soft surface material is lost to brush abrasion. These SEM results are also consistent with the calcium dissolution from swishes in **Figure3**.

4.4.4 CLINICAL CASE REPORT [SEE FIGURES 4-12(A – J)]:

Locating people with ‘fad diets’ fixated on chronic consumption of carbonated drinks is extremely rare. It would be considered immoral and/or unethical to limit volunteers’ diets, and/or force or restrict liquid intakes which cause damage to their teeth. However occasionally an individual, for obscure reasons, will abuse liquid intake and restrict themselves to one type of drink. Accordingly, included here is a rare case report of cola abuse and its’ effect on teeth. The case consulted initially at an oral medicine clinic. The rarity of showing how a full dentition could be affected from long-term cola abuse warrants it being included in this study, as it shows the (admittedly accelerated) practical results from imbibing acid colas.

An 18 year old male presented complained of temperature sensitive teeth, and that his “teeth were getting smaller” His medical history did not display symptoms suggesting that he may suffer from GORD. (Gastro Oesophageal Reflux Disorder).The aetiology of GORD is variable but when the gastric contents flow from the stomach to the mouth, it is called Gastro-oesophageal reflux disorder (GORD) (22). Common symptoms are: Heart-burn along the oesophageal pathway; epigastric pain localised over the stomach; regurgitation into the mouth; dysphagia with or without pain; non-cardiac retro-sternal pain; chronic coughing and sore throat from laryngitis; vocal hoarseness; and a throat globus (51;52) None of these symptoms or signs were reported by the patient. He had regular check-ups with his general dentist, and every day brushed and flossed his teeth regularly morning and evening. He used fluoridated toothpaste and changed to a new soft nylon tufted tooth brush every month. As for his diet, it was based on foods in all food groups [meat/fish, dairy, grain/cereals, vegetables] but he expressly emphasized he did not drink fresh fruit juices and infrequently ate fresh fruit because “they hurt his teeth.” However, he reported drinking abundant amounts of cola “solidly from my last two years of in primary school till now”. A period of time was calculated to be approximately ten years. His consumption consisted of all brands of conventionally [carbohydrate] sweetened colas, until three years prior to presenting, when he switched to drinking exclusively synthetically sweetened “diet-colas”. A two week dietary analysis confirmed his high cola intake was indeed between at least one-litre to one-and-a-half litres of cola daily, from either cans or bottles, depending on availability. This

daily cola consumption was habitual part of his diet and he “swished the cola over his teeth to reduce the gas and to enhance the flavour.”



FIGURE 4-12A



FIGURE 4-12B



FIGURE 4-12C



FIGURE 4-12D



FIGURE 4-12E

FIGURES 4-12(A, B, C, D, & E): The teeth are smaller, with shiny surfaces. The full inter-cuspal occlusion displays spaces from reduction of cuspal height; the incisors, canines and premolars are eroded palatally, and worn down with attrition.



FIGURE 4-12F



FIGURE 4-12G

FIGURES 4-12(F & G): Occlusal views of Upper and Lower arches. Loss of occlusal tables from attrition and erosion is evident on the premolars and molars; there is occlusal saucerisation of cusps on the first and second molars.



FIGURE 4-12H

FIGURE 4-12H: Pre-op view of upper anteriors. Note short vertical height of incisors, and loss of buccal enamel on all upper teeth.



FIGURE 4-12I



FIGURE 4-12J



FIGURE 4-12K

FIGURES 4-12 (I, J, & K): Healing post-op view after clinical crown-lengthening. The central incisors are longer, the premolar palatal cusps are lost and the palatal aspects show a clear palatal step where enamel has eroded away. This erosive pattern involves the buccal surfaces of the upper teeth, and is decidedly different when compared to the erosive patterns encountered with GORD(52).

On presentation **[Figure 4-12 A-K]** his teeth appeared reduced in size apparently by erosion with reduced enamel and dentin exposure. The upper anteriors were more affected than the rest of the teeth, along with molar and premolar cuspal reduction and incisal attrition. The patient felt pain when gentle air-stream was passed over his incisors. All the teeth reacted to thermal stimulation were vital. Staining of the teeth confirmed excellent oral hygiene [plaque index- modified O'Leary- below 5% of surfaces]. Sub-gingival probing revealed no periodontal disease [no sulcus depth

exceeding 3mm] and further sub-gingival exploration showed only healthy tooth surfaces and no caries.

This case report reflects dental damage from abusive cola drinking, and the clinical presentation can be explained from data presented in this thesis. The chronic regular consumption of low pH cola drinks encouraged the erosion of the teeth. The loss of anatomy and sensitivity are direct results of acid cola dissolving coronal tooth material. The gross presentation clarifies what the microscopic loss will result in over time. The SEM figures [4-6 to 4-11] shows microscopic erosion, which when repeated frequently and long enough, will manifest like this clinical case of cola-erosion.

4.5) Discussion

4.5.1 pH, ACIDITY AND BUFFERING

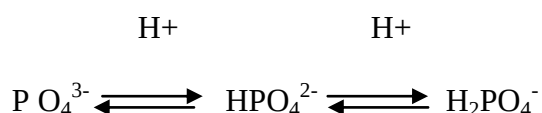
Each of the standard cola-drinks and diet Cola beverages reveal a progressive increased requirement of base to neutral pH 7. But the pH and buffering does not fully explicate the erosive capability of colas, as the mineral content, concentrations of organic acids [phosphoric and citric], fluoride and the ability of the mix to remove calcium from the mineral surface are contributing factors (20). However, pH expressing acid content, buffering ability, and acidic ions available for the overall general mordant effect of acid-cola beverages are more important in producing erosion, as without an acid environment the other stated ions are not active. **Figures 4-1** indicates just how low the pH value of the selected group of pop Cola-drinks are. All the cola-drinks register pH values significantly below the critical pH 5.5, the point at which tooth decalcification occurs [**Figure 4-1**]. Of all the tested drink, Pepsi-Cola is found to be the most acidic [pH2.53] while least acidic is Diet

Selection-Cola [pH 3.4]. As noted in **Figure 4-2**, Selection-Cola and Diet Pepsi-Cola present the largest buffering capacities giving a pH change of one unit.

In order to change up the pH level to its critical value pH5.5 [**Figure 4-2**], Pepsi-Cola demands the largest amount of sodium hydroxide, while Diet Pepsi-Cola [**Figure 4-2**] displays the most resistant drink to a pH change up to neutralization [pH 7.0]. These finding are notable because the low pH values confirm that pop Cola-drinks are highly acidic and therefore may advance the delcification of teeth. [**See Figures 4-6 to 4-11**]. Knowing this, to raise the pH back to the neutral level of pH 7.0 it is necessary to obtain an average of 5.86mL of base [**Figure 4-2**]. Therefore this proves cola drinks are highly acidic when they are first exposed to teeth, but require large amounts of alkaline stimulated saliva to be neutralized.

4.5.2 RINSING WITH AND WITHOUT TEETH IN VIVO

Though whole saliva can function as a complex buffering solution providing a definitive protective buffering and neutralizing effect on acid dietary drinks (39-41), but with acid drinks including pop colas, this buffering/neutralizing effect may be overwhelmed (42). Saliva is saturated with calcium and phosphate as tricalcium phosphate, octa-calcium phosphate, and less often dicalcium phosphate dehydrate [$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$]. Teeth contain phosphate as PO_4^{3-} in their HA crystals, but this ion cannot exist in solution at physiological pH values except in minute amounts. When this ion is released from HA crystals into a medium, it is changed to HPO_4^{2-} , which converts quickly to H_2PO_4^- when the pH drops to about pH 3



Accordingly as the pH falls, these changes increase, and lowers the concentration of PO_4^{3-} , and more PO_4^{3-} ions release from the HA. The concentrations of calcium and phosphates influence the rate at which HA dissolves by the law of mass action. The pH at which any oral solution ceases to be saturated is referred to as the critical pH, below which the inorganic matter (calcium hydroxyapatite) of teeth will dissolve in it (53). The low pH of cola renders the oral environment unstable and phosphate, and calcium ions are released.

While the colas tests are not all chemically identical, they all caused calcium to leach from teeth *in vivo* after contact for 60 seconds **[Figure 4-3]**. Due to the varying level of phosphorous content of the colas from the cans, phosphorous levels will differ. The average phosphate in resting whole saliva (as P) is accepted at 16.8mg/100ml, and for stimulated whole saliva 12 mg/100ml (53;54). The range of phosphate (as P) is 6.1-71mg%, as the flow and contents from the parotid, submandibular and minor salivary glands varies, and there are also rapid and slow secretor (53-55). The effect of stimulation in reducing phosphate and calcium in whole saliva is exceptional, because there is an increase in flow from the parotid and the change in phosphate concentration is a real reduction in the secretion from the glands(53). The average flow rate is about 20ml per hour (slow secretors 13.4ml, and rapid secretors 39.6ml per hour)(53;56). So that the amount of active whole saliva stimulated in one minute was assumed not to exceed 2ml.

The concentration of stimulated whole saliva during 30 and 60 seconds of swishing cola was considered as 12mg/100ml, as stimulated saliva has a lower concentration

of phosphate, and the stimulating times were low(53). The total amount of phosphorous secreted in one minute from saliva, contributed to the P swish measurements, and was calculated to be small...about 0.24mg. This small amount was discounted and regarded not as a source of phosphate influencing erosion. Consequently phosphorous was not measured in saliva, other than that found in the control swish with water.

Phosphoric acid is an added main constituent in all the colas tested. This is the main source of Phosphorous in the swishes **[Figure 4-4]**.

The dental and salivary calcium salts does provide absorption of phosphorous from the cola. Additionally, the acidity from each cola varies as does the buffering and reflex stimuli may vary in intensity of reaction. Other intra-oral phosphate sources may derive from gingivo-crevicular-fluid, and calculus (tricalcium phosphate, octa-calcium phosphate, dicalcium diphosphate). Therefore, as of the cola phosphorous composition levels are variable, the amount of phosphates produced after swishing is also variable **[Figure 4-4]**. Even though the chemical composition of the colas tested differed from one another, they all presented high buffering capacities and acidity [pH] well below the critical pH5.5.

When comparing calcium content between the swished expectorates and the calcium content in the cans, swished expectorates reveal consistently and crucially [$p<0.05$] higher calcium levels than those found in the can, even when considering the presence of extra calcium released in stimulated saliva (15). Tooth erosion, as chemical dissolution of calcium, derives from an intra-oral source, and is reflected by an increased content of calcium in all the swishes from the colas tested. However,

the increase in calcium shown in the swishing experiment *without teeth* [Figure 4-3] after rinsing with the colas could be present due to calcium ions secreted in stimulated saliva. Additional sources of calcium may occur in miniscule aliquots from, minor salivary glands, and circulating oral glycoprotein. Though the calcium content in expectorates vary, *the only confounding factor as a variable that explains the increase of calcium in the swish expectorates is the presence of teeth*. This situation is explicated by comparisons in each cola of calcium content in the expectorate *swish-with-teeth* that show significant [$p < 0.05$] increases in dissolved calcium and differing from the swishing-with-water, or *swishing-without-teeth* test. It is possible phosphates can derive from stimulated saliva, and some from the teeth or other intra-oral phosphate sources. But the amounts of calcium contents measured in the swishes cannot be explained in this experiment, from sources other than from the natural teeth *in vivo*. Taking into consideration that average rate of resting-saliva secretion is 0.78 ml/min, it would take a significant length of time for the mouth to return of neutral pH7 (48;49). Accepting the role of acids as most potent stimuli for reflexive stimulated-salivary flow, it is possible stimulated secretion can increase to reach a maximum limit of 8ml saliva per minute (20). Yet even if this maximum rate is reached when consuming cola-drinks, it still would take a lengthy period of time for neutralisation to physiological stable oral pH levels to be achieved. Furthermore, the calcium content from saliva is too low to account for calcium increases after rinsing. Typically after one test-bolus in the mouth this is about 25 to 30 minutes (48). At 20 mg/L F^- fails to reduce erosion of teeth *in vitro* (41). Even with the mean fluoride content from the cans at 3.5 mg/L [ranging from 2 to 6 mg/L]; this

concentration does not arrest calcium dissolution post swishing. The amount of fluoride found in saliva is very low; amounts of 0.02ppm, ranging between 0.01 and 0.05 ppm (in the reactive ionic form) has been measured using a single fluoride electrode(57). Accordingly fluoride was not measured in the saliva of subjects. Fluoride reacts with calcium ions to form stable CaF which dissociates at pH below pH 3.5(58;59). The calcium ions available in plaque or on the tooth surface react with salivary fluoride; the fluoride binds with organic molecules like calcium phosphate, or calcium hydroxyapatite. The fluoride content reported here, (**see figure 4-5**) seems high with the techniques used. The low salivary fluoride was not considered a confounding factor because the amount of calcium released after swishing with cola increased in spite of the high fluoride content in the colas. The high fluoride content is probably added to moderate decalcification. However, the low cola pH may cause some bound precipitated CaF to dissociate and release calcium and minute amounts of fluoride into the swish. The total acidity of the colas overwhelms any moderating effect the fluorides may have, as after swishing the calcium released from teeth is augmented. Fluoride in resting and stimulated saliva is minimal (0.02ppm F^- , 0.01 F -ppm, respectively) [**See Figures 4-5**]. It could be possible to increase the fluoride content; however, higher concentrations are undesirable due to the unwanted toxicity and would negatively affect organoleptic taste properties. It is clear that dietary acids from colas overpower any potential protective abilities found in saliva. The same consideration applies when considering fluoride content of the colas.

There are other important biological factors that may slightly affect decalcification and tooth erosion, such as the saliva flow rate and its composition, buffering capacity and stimulation capacity; and the acquired pellicle, which has diffusion-limiting properties by its composition, maturation and thickness (60). Additionally, the type of dental substrate and its density of composition also can effect erosion, as does the dental anatomy and occlusion influencing the flow of liquids over the tooth surfaces. Besides the anatomy and histology, the vigorous function of oral soft tissues in relationship to the teeth also affects the development of erosion(60). These causal factors considered none can be determined to be as important as the acid composition and pH of the pop-cola drink in producing erosion.

Furthering erosion, it appears that Keratosis on the tongue acts as a rasper that removes surface tooth material softened by decalcification. Decalcification caused by regurgitated gastric contents in bulimia, often manifests first as palatal erosion because of tongue thrusting, removing softened tooth material (60-62). The acidulated colas also act as a stimulus for stimulated saliva to flow which contains calcium. But the calcium content of saliva is negligible [mean 5.7 mg/100mL; range: 2-10mg/100mL], and *even with maximum secretion rate of saliva with ranges at 7 to 8 mL* in one minute (63), comparisons between swish expectorates [*with and without teeth*] indicate it is impossible for stimulated saliva to secrete calcium in amounts recorded after 60 seconds of swishing. Due to the inordinate difficulty to procure age matched [edentulous] controls without teeth below 35 [dentate controls with teeth had a mean age 22 years old], or to locate people at aged 52 years [the mean

age of the test edentulous group] without any dental restorative work: age was discounted as a confounding factor in the comparison.

4.5.3 SCANNING ELECTRON MICROSCOPY (SEM):

The growth of consumption of pop-drinks in recent years (42) is reflected in increased reporting of dental erosion (43;44). Data reported here shows damage occurs at a microscopic level and corroborates information sourced from epidemiology and marketing studies. (43). Some consider a 30 or 60 second exposure unreasonably long; however, this is not valid criticism, as most reported data from other investigators use target-times in excess of 5 minutes, even hours or days of immersion (64). The test periods of 30 seconds and 60 seconds could well approximate the total time a 350ml can of cola may be exposed to teeth, when people swallow a 60ml bolus and swish it for 5 to 10 seconds. Evidence indicates dental ravages from cola drinks have a high prevalence in children and young adults (45;46). Evidence presented here in this electron microscopic study re-enforces and confirms this theory. Furthermore, this study demonstrates this interaction results in surface etching [**Figure 4-6**] and cracks [**Figures 4-8 &4-10**]. This SEM evidence corroborates observations regarding smear layer removal, opening of dentine tubules, and increasing of tubules diameter based on randomly selected sites (65;66). Exposure of dentinal tubules by acidulated colas may result in dentine hypersensitivity (66). This is probably from disruption of fluid dynamics in the tubules, as well as by mechanical loss of tooth material (65;66). The results also indicate that with light brushing after 60s exposure to pop-colas, abrasion will occur.

4.5.4 CLINICAL CASE REPORT

Tooth wear results for three main processes erosion, attrition and abrasion, with chemical, physical and physiological forces interacting to produce a clinical case of dental damages. The case presented displays all three major effects **[Figure 4-12]**. Saliva might temperate these damages through pellicle formation and remineralisation processes. But these protective influences are overwhelmed by frequent drinking of pop-colas and causing damages. Restorative therapy for dental damages is possible but will vary depending on the extent of damage. Therapy ranges from avoiding the consumption of acid-drinks, refraining of brushing immediately after drinking pop-drinks, reducing frequency of drinking, to fissure sealants and coating, occlusal build-up with overlays, or comprehensive oral rehabilitation with full coverage crowns (67) .

4.6) Concluding Remarks

[i] Pop-Cola drinks tested are shown to be highly acidic and can potentially decalcify tooth material.

[ii] This study provides evidence that all these six common colas (Pepsi-Cola, Diet Pepsi-Cola, Coca-Cola, Diet Coke, Selection-Cola and Diet Selection-Cola); leach calcium out of teeth, after rinsing with pop-colas.

[iii] This SEM experiment reveals that acid pop-cola ignores the smear layer, softens and erodes dental hard tissues, and facilitates abrasion.

[iv] The clinical case report shows erosion from chronic imbibing of acid pop-colas. These data collectively provide further evidence as proof that chemical dissolution by tooth decalcification is caused from the consumption of pop-colas. This study provides and demonstrates clear visual evidence of dental erosion with altered enamel and dentine morphology changes due to short exposure from pop colas.

4.7) Declaration: Contributions by authors of the publication

Amirfirooz Borjian: Wrote first draft of the paper, edited all preparation manuscripts, collected, retrieved and prepared the samples for assessment, wrote interpretation of results, did most of the chemical analysis, SEM analysis and helped locate and describe the clinical case.

Claudia CI Ferrari: Contributed to the writing of the introduction, interpretation and discussion sections of preparation-scripts, helped collect, retrieve and prepare the samples, assisted with the chemical analysis, and describe the SEM analysis and clinical case.

Antoni Anouf: Contributed to the writing of the scripts, helped collect, retrieve and prepare the samples, assisted with the chemical analysis, and describe the SEM analysis and clinical case.

Louis Z. G. Touyz: Initiated the project, supervised, co-ordinated, motivated and criticized the Beverages and Teeth Team (AF, CICF and AA above); edited and contributed to the interpretation and writing of the scripts. Supervised collection, retrieval and preparation of all samples, and assisted with the chemical analysis, and the description of the SEM analysis and clinical case. He is the corresponding author of the article.

Chapter 05

Man-Made Alcoholic and Non- Alcoholic Beverages

(Future Research Projects)

Outline

Introduction

-man-made alcoholic beverages (MAM-ABS)

-man-made non-alcoholic beverages (MAM-NABS)

Complexing agents

-EDTA

-Citric Acid

Experimental Limitations

Improvement of the Experiments

5.1) Introduction

Production and consumption of man-made alcoholic beverages (MAM-ABS) and man-made non-alcoholic beverages (MAM-NABS) is now a global industrial economic activity. Various agricultural resources, harvests and crops provide annual renewable raw materials that fuel the beverage industry. This resource avoids waste and benefits farmers, consumers and world economies. Organic (fresh fruits, roots, botanicals, vitamins, sugars) and inorganic (chemically constituted acids, carbohydrates, synthetic sweeteners, ascorbic acid) provide raw materials sources or supplement as additives in the manufacture of beverages. Other naturally sourced MAM-NABS like tea, coffee or cocoa, all contain natural flavourings as well as pharmacologically active drugs. Substances like caffeine, pheobromine and theophyllin are prevalent in drinks made from these sources. Alcoholic beverages (mead, beer, wine, ciders) are strictly controlled. Both MAM-ABS and MAM-NABS exploit the gaseous chemistry with flavourings but many MAM-NABS now include pharmaceutically active stimulants like caffeine. Because, the chemistry of gaseous soda water with added flavourings is well understood, most of these beverages, both MAM-ABS and MAM-NABS, are presumed to be innocent sources of nutrition and calories. While most think of these beverages as health drinks (especially ‘natural juices’ and fruit flavoured soft-drinks) with general nutritional benefits, universally little attention or consideration is focused on what effects these beverages, both MAM-ABS and MAM-NABS, have locally on organs and on teeth. All these drinks should be investigated not only with regard to general health, its relation to other causes of erosion (50) and locally on the gastro-intestinal tract, but also specifically

what effects they have on teeth. Beverage companies wish to have the consumption of their drinks increase, because this augments their financial profits. Spurious marketing strategies need to be subject to stringent controls in the interests of public health. For example in “Fuze” (Vit-A is added to Cola), sold as a health drink, may result in communal health problems.

5.2) Complexing agents

EDTA or other acids (like benzoic, or carboxylic fruit acids) substances would act as chelating agents and should be avoided to add into the drinks. EDTA is a strong chelator and it is not included in pop-cola beverages. It will demineralise calcium even at neutral pH, because of the strength of its combining with calcium ions(20).

Citric acid is even more complex, as it has 3 pKa values, one for each of the H⁺ ions reversibly bonded to the citrate ion. Citric acid added may crystallize out or preferentially precipitate calcium; consequently added calcium to drinks could inhibit the chelating effect of that acid(20). This reaction is most prevalent in fruit juices. Most novel chemical combinations will negatively affect organoleptic properties with off tastes. This is why few cola imitations (Pepsi is the exception) have produced serious competition to Coca-Cola. The effective taste is the result of a perfect proportioning of caramel, phosphoric acid, citric acid, sweeteners, sodium benzoate, and caffeine.

5.3) Experimental Limitations

In this study procuring age matched cohorts was nearly impossible. Commercial potable carbonated drinks used, because of consumer usage and availability; but

gasification is unreliable and variable. All experiments were done with flat (degassed) drinks in vitro. This may lead to slowing down of chemical reactions and under reporting of the results. All experiments were done at room temperature (25°C); many carbonated beverages are consumed at temperatures below 10°C.

When considering larger sample sizes of cohorts, volunteers' drinking style, and testing in variable temperatures, a low temperature may impact accuracy of the results.

When dissolving NP-GHA crystal in a cola, measures by mass to determine amounts of calcium lost demands highly sophisticated techniques and use of bigger quanta of test substances. The major confounder is because the cola contents reacts with the NP-GHA, and measures of loss of calcium demands methods other than simple assessments by mass measures.

5.4) Improvement of the experiments

Possible extensions of this study: experiments such as in vivo tests with deciduous teeth, assessing effects of carbonated drinks in children are indicated. Measures of fluoride could be improved (using a single Fluoride Electrode), in saliva in groups, with and without teeth, before and after rinsing... for better clarification of the effect of fluoride in colas influencing erosion on teeth. By using other combinations of specific techniques such as:- ... surface hardness measurements, surface profilometry, iodide permeability tests, chemical analysis of dissolved minerals, micro-radiography, confocal scanning microscopy, quantitative light induced fluorescence, atomic force microscopy, element analysis of solid samples, nano-indentation, and/or ultrasonic measurements,... all could be employed to enhance producing useful results,

identification and quantification of trace element, and the effects of acidulated beverages on teeth. Also more details about acid contents, types of acids, acidity and all additives need to be placed on consumer bottles, to allow consumers to make informed choices. More research into these drinks is needed.

Appendix

-Descriptions for technique details of ICP-OES and IC -Statistical Analysis

Appendix I

pH MEASUREMENT:

The Automatic Titrator Instrument was standardized with certified reference buffers at pH 2, 4, 7, and 10. A combination electrode was used to measure both the reference materials and the colas. All measurements were completed at a measured temperature of 23°C. Each sample was measured 12 times.

Appendix II

INDUCTIVELY COUPLED PLASMA-OPTICAL EMITTANCE SPECTROSCOPY (ICP-OES):

For the purpose of our analysis, the ICP-OES was used to detect the concentrations of *calcium, phosphorus*, (and sodium, potassium, boron, tin, arsenic, iron, nickel, lead, copper, magnesium, zinc, sulphur, chrome, cobalt and manganese.. not reported here) present in the cola drinks being tested. In order to obtain accurate and precise results, twelve replicates for every drink being tested along with a de-ionized Aquafina water blank were prepared with due diligence. Initial Pre-cleaned Polypropylene *DigiTUBE*s (50 ml) were clearly labelled, and 20ml samples were dispensed to each tube with a pipette. Because there is a high sugar content in the regular drinks, a matrix effect is formed, preventing the ICP-OES from determining accurate concentrations of those elements being tested. To eliminate any potential

matrix, 5 ml of Trace Metal Grade Nitric Acid was added to each sample, and the samples were digested to 95°C for 120 minutes using a 24 position *DigiPREP* equipped with a *DigiPROBE*. The probe was placed in a sample in order to maintain and monitor the digestion procedure. Once digestion was finished: 1) The samples were cooled to room temperature; 2) Volumes of twelve replicates and a blank was completed to 50 ml with the de-ionized water.

The same procedure was performed for each of the six cola-drinks being tested. A calibration curve was prepared from 0.5 to 50 mg/L with certified ICP-OES standards diluted in a solution of 2% Nitric Acid. A total of four points including a calibration blank (consisting of 2% Trace Metal Grade Nitric Acid) were prepared. Furthermore, two Quality Control Standards were prepared from a different lot of certified ICP-OES standards. They were prepared at a concentration of 5 and 25 mg/L.

Appendix III

ION CHROMATOGRAPHY (IC):

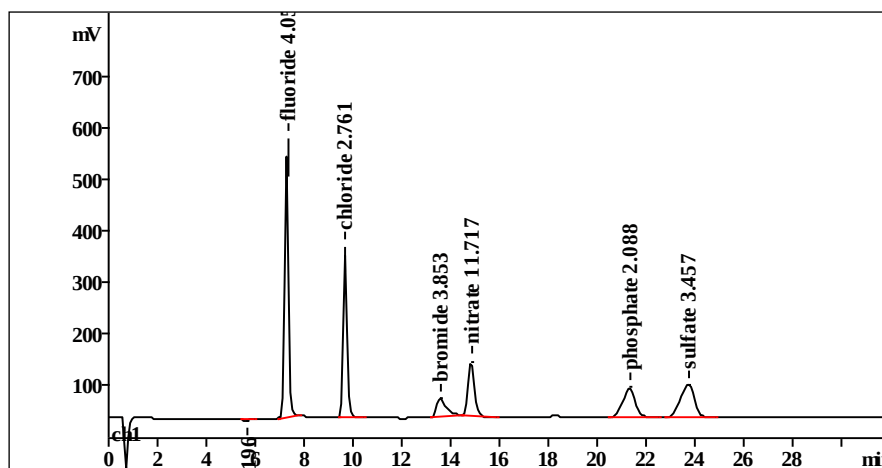
The IC is used to determine the concentration of different anions present in the six cola drinks being tested. Even though a comprehensive analysis for fluoride, acetate, formate, chloride, phosphate, nitrate and sulphate, is feasible, for the purpose of our analysis, the concentration of anion fluoride was studied. The other ions assessed are not reported here.

A fixed volume (5 ml) of the cola is accurately delivered in a properly labelled *DigiTUBE*s (50 ml). The cola samples were then filtered using a very small filter (diameter of pores = 0.45 μ m) to remove all sediments and impurities present

that would cause microbial alterations. A volume of 4ml of every filtered sample is then collected in a container using a well rinsed polypropylene vial.

A calibration curve was prepared using certified IC standards for all analytes. The calibration curve covered a range from 0.01 to 10 mg/L for each anion. Furthermore, two quality control standards were used at concentration of 0.02 and 5 mg/L **(See Appendix Figure-A)**.

Below shows an output of an ion chromatograph for a multi-standard solution. Each peak on the graph represents a different ion. The species in the solution can be identified by their elution time. The concentration of each ion can then be obtained by evaluating the area under each peak. The concentration of the anions in the cola drinks were determined by plotting calibration curves.



Appendix Figure-A: IC output for multi-standard solution. The concentration of the different ions can be obtained from the ion chromatograph

Appendix IV

All analyses were done using the SAS, version 9.2 (SAS System, Inc., Cary, NC).

All statistical tests were two-sided.

P-values <0.05 were considered evidence of statistical significance.

FIGURE 3-1: pH COMPARISON AMONG THE DRINKS

P-values:	Aquafina Water	Gassified Pepsi	Degassed Pepsi
Aquafina Water		<0.0001	<0.0001
Gassified Pepsi			0.1499

FIGURE 3-2: BUFFERING CAPACITIES OF SELECTED DRINKS UP TO pH7

P-values:	Aquafina Water	Gassified Pepsi	Degassed Pepsi
Aquafina Water		<0.0001	<0.0001
Gassified Pepsi			0.0012

Table 01: Result from One way Analysis of Variance (ANOVA)

Figures 3-3 & 3-4	Overall F-Test	p-value
Calcium	F(3,20)=36.9	<0.0001
Phosphorus	F(2,15)=109.2	<0.0001

Figures 3-5 & 3-6	Overall F-Test	p-value
Calcium	F(3,20)=19.5	<0.0001
Phosphorus	F(2,15)=9.3	0.0024

Table 2: Mean, Standard Deviation and Adjusted P-values of Pairwise Post-hoc comparisons of means

Variable	Mean and Standard Deviation							
	Figures 3-3 & 3-4				Figures 3-5 & 3-6			
	Calcium		Phosphate		Calcium		Phosphate	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Aquafina Water	3.35	1.14	1.83	0.6	1.51	1.47	1.33	1.59
Neutral Pepsi	6.49	0.33	138.58	2.82	1.91	1.45	120.16	2.98
Gassified Pepsi	9.67	1.06	148.67	2.13	18.51	8.94	184.41	40.65
Degassed Pepsi	9.41	1.78	122.04	4.16	14.23	16.05	147.24	18.84
Variable Comparisons	P-values*							
Aquafina Water Vs Neutral Pepsi	0.0012				0.8166**			
Aquafina Water Vs Gassified Pepsi	<.0001				<.0001**			
Aquafina Water Vs Degassed Pepsi	<.0001				0.0004**			
Neutral Pepsi Vs Gassified Pepsi	0.0011		<.0001		0.0003**		0.0005	
Neutral Pepsi Vs Degassed Pepsi	0.0024		<.0001		0.002**		0.1962	
Gassified Pepsi Vs Degassed Pepsi	0.9804		<.0001		0.6486**		0.0475	

*Adjusted p-values obtained for the bootstrap resampling of the residuals.

** Log-transformed values of Calcium

Table 03: Mean, Standard Deviation and Adjusted P-values for each group (with teeth & without teeth) per drink [Figures 4-3 & 4-4]

Drink	Teeth	Calcium				Phosphorus		
		Mean	Std Dev	P-value *		Mean	Std Dev	P-value *
Pepsi	No	1.19	0.98			157.20	7.32	
	Yes	4.15	0.97	0.0036		149.00	2.56	0.0215
Coke	No	3.81	0.71			157.20	5.35	
	Yes	14.10	1.20	0.0004		84.56	4.01	0.0005
Cola Selection	No	22.34	1.36			189.30	2.16	
	Yes	43.99	5.68	0.0004		182.80	4.91	0.0168
Diet Pepsi	No	1.72	1.16			165.90	2.88	
	Yes	4.42	1.11	0.0036		149.10	11.15	0.0124
Diet Coke	No	5.51	1.02			85.36	6.00	
	Yes	12.14	0.68	0.0004		40.77	2.61	0.0005
Diet Cola Selection	No	25.16	1.49			131.70	3.74	
	Yes	28.62	1.57	0.0036		123.00	4.77	0.0168

* Hochberg's adjusted p-values

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