

Antimicrobial and Cleaning Modes of Action of Peroxide and Reactive Oxygen Species for Combating Biofilms

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Declaration

I, Cláudio Lourenço, hereby confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Poor denture hygiene is the underlying cause behind several undesirable effects including denture stomatitis, bad breath, staining among others. Dentures are immediately exposed to microbes and quickly become colonized by biofilm. Dentures therefore require regular cleaning and disinfection by a safe and reliable product. With this in view, the effects of reactive oxygen species (ROS) in the removal of biofilms and stains were studied. Furthermore, the kinetics of the reaction and the pH conditions necessary for effective production of reactive oxygen species within a formulation and its antibiofilm properties were also investigated.

$^1\text{H-NMR}$ was used to kinetically follow ROS production at different pH in the tetraacetylenediamine (TAED)/ hydrogen peroxide (H_2O_2) system within different formulations and complemented with fluorometric detection of ROS.

Subsequently the antimicrobial properties of the tested formulations were observed against oral microorganisms in suspension and were supplemented with biofilm studies to visualize the biofilm removal properties of ROS using confocal microscopy and scanning electron microscopy.

Quantitative $^1\text{H-NMR}$ and fluorimetry proved to be robust and reliable methods to assess the efficacy of a formulation in delivering ROS. At pH 5.5, the reaction progressed more slowly but generated a higher amount of singlet oxygen while at pH 7 the initial reaction occurred slightly faster, but the generation of ROS was slower.

ROS were also a key component in the removal of biofilm. When Polident was tested vs a placebo tablet it was observed that Polident not only removed biofilm but also killed nearly all of the remaining cells while placebo only managed to remove some biofilm and left many live cells. At optimal pH with only ROS as a weapon our novel formulations achieved results comparable with the denture cleanser tablet proving the excellent antimicrobial properties of ROS.

Impact Statement

Dentures are more common than we may expect, according to the Oral Health Foundation 16% of the total population of the UK wear dentures, and almost a million of them are people aged between 16 to 44. The WHO also mentions that the number of patients requiring dentures worldwide is on the increase.

Denture users experience complications such as toothache, painful gums and sore spots that lead to chewing and biting problems affecting their diet and nutrition, also some people feel embarrassed and sometimes avoid conversations and participation in social activities due to the state of their dentures. Recent research also points to the fact that some oral diseases share some risk factors with cardiovascular and respiratory diseases, cancers and diabetes.

The oral cavity is the habitat of one of the most complex biofilms known to man, the dental plaque. There are up to 700 different microbial species in the oral microbiota colonizing virtually all oral surfaces.

But not everything about the oral microbiota is undesirable; a mouth with a “healthy” biofilm does not present any case for concerns but an imbalance in the oral biofilm composition or the introduction of exogenous microorganisms can lead to adverse effects and disease. One such condition is denture stomatitis associated with an increase in the fungus *Candida albicans*.

Therefore, for denture wearers additional care with their oral hygiene becomes a priority. Considering that normal toothpastes and brushes are not recommended for this task it is of great relevance that development of enhanced denture cleaning formulations are a priority to achieve a better and more effective denture hygiene.

With this in view this project was aimed at studying a current formulation and understanding the kinetic mechanisms behind the generation of a cleaning and antimicrobial agent, peracetic acid. Peracetic

acid is a powerful bleaching and antimicrobial agent with a proven track record of success. It has several applications ranging from a disinfectant in hospitals to a bleaching agent in household laundry.

This study was based on the generation of peracetic acid through the acylation of tetraacetythylenediamine by hydrogen peroxide. Once in solution, at the right pH, peracetic acid can generate singlet oxygen, a powerful reactive oxygen species.

This multidisciplinary project involved spectroscopy, fluorimetry and microbiology and will impact with the development of a quick reactive oxygen species screening method to be applied to future formulations based on quantitative $^1\text{H-NMR}$ and indirect fluorometric measurements. The formulations developed during this study proved that reactive oxygen species are an important factor to consider when antimicrobial efficacy is our goal. The formulations developed were effective in removal of biofilm and killing of bacteria but were based on three ingredients without considering any other formulations requirements such as anti-caking agents, surfactants etc.

The correlation between systemic diseases and oral diseases brings once more the spotlight on the importance of maintaining good oral health and our research aims to help people that already have their oral health compromised.

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List of Abbreviations and Symbols

| | |
|-------------------------------|--|
| °C | Degree Celsius |
| ¹ H-NMR | NMR Proton Nuclear Magnetic Resonance |
| AA | Acetic acid |
| BHI | Brain Heart Infusion |
| CA | Citric acid |
| CFU | Colony forming units |
| D ₂ O | Deuterium oxide |
| DAED | Diacetylenediamine |
| DAP | Diacylperoxyde |
| DCFH | Dichlorodihydrofluorescein |
| H ₂ O ₂ | Hydrogen peroxide |
| HA | Hydroxyapatite |
| MBC | Minimum Bactericidal Concentration |
| MBEC | Minimum Biofilm Eradication Concentration |
| MIC | Minimum Inhibitory Concentration |
| MPS | Potassium monopersulfate |
| NaPC | Sodium percarbonate |
| NF | Not Found |
| NOBS | Nonanoyloxybenzene |
| PAA | Peracetic acid |
| PBS | Phosphate Buffered Saline |
| PEMA | Polyethyl methacrylate |
| PI | Propidium iodide |
| PMMA | Polymethyl methacrylate |
| qNMR | Quantitative Nuclear Magnetic Resonance |
| ROS | Reactive Oxygen Species |
| SEM | Scanning Electron Microscopy |
| SG | Singlet Oxygen Sensor Green |
| CLSM | Confocal Laser Scanning Microscopy |
| SLSOA | Sodium Lauryl Sulfoacetate |
| SO | Singlet Oxygen |
| TAED | Tetraacetylenediamine |
| TBCC | N-[4-(triethylammoniomethyl)-benzoyl]-caprolactam chloride |
| Tri-AED | Tri-acetylenediamine |
| ve- | Negative Control (No growth) |
| ve+ | Positive Control (maximum growth) |

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1. Introduction

1.1 Denture Concerns

Dentures are not only a concern for the elderly people. Despite being often invisible among the population, dentures and denture wearers are more common than what we see or think on a daily basis. According to the Oral Health Foundation there are 11 million denture wearers in the UK, 16% of the total population, and almost a million of them are people aged between 16 to 44¹. In the European Union 31% of the population wear removable dentures².

Table 1 Percentage of people who use removable dentures in Europe. Table adapted from the Special Eurobarometer 330 Report on Oral Health²

| Denture users in Europe | | |
|--|-----|-----|
| | Yes | No |
| EU27 | 31% | 66% |
| Gender | | |
| Male | 27% | 71% |
| Female | 36% | 61% |
| Age group | | |
| 15-24 | 4% | 92% |
| 25-39 | 5% | 93% |
| 40-54 | 18% | 80% |
| 55 + | 50% | 47% |
| Experience difficulties eating food | | |
| Yes | 41% | 56% |
| No | 29% | 68% |
| Felt embarrassed because of their teeth | | |
| Yes | 39% | 58% |
| No | 31% | 67% |

According to the Special Eurobarometer Oral Health denture wearers experience complications such as toothache, painful gums and sore spots that lead to chewing and biting problems affecting their diet and nutrition². It has also been reported that some felt embarrassed and sometimes avoid

conversations and participation in social activities due to the state of their teeth and mouth².

Oral diseases share some risk factors with chronic diseases (cardiovascular and respiratory diseases, cancers and diabetes) hence the importance of a good oral hygiene²⁻⁴. The more widespread and recognizable risk factors are poor hygiene of the mouth, smoking, alcohol abuse, poor nutrition as well social factors⁵.

The mouth cavity is the habitat for numerous species of microorganisms (bacteria, fungi and viruses)⁶⁻⁸, there the majority of microorganisms grow as a biofilm also known as dental plaque^{7,9,10}. There are over 700 different microbial species in the oral microbiota colonizing all surfaces in the mouth cavity^{6,9,11}. A healthy mouth with an “healthy” biofilm normally does not present any problems but an imbalance on the biofilm present or the introduction of other microorganisms can lead to adverse problems and disease¹². Recent research suggests that the oral cavity microbiome can contribute to some systemic and chronic diseases¹³ but also more commonly denture stomatitis and mal odour¹⁴.

Dental materials when exposed to the oral environment become quickly covered by saliva constituents and then colonized by the oral microbiota¹³⁻¹⁵. The introduction of a full or partial denture will drastically increase the surface area of the mouth cavity and consequently, the number of microorganisms will also increase^{13,16,17} as well the incidence of caries and oral disease¹⁸. So, for a denture user additional care in the oral hygiene are mandatory and not only an option. Normal toothpastes and toothbrushes are not recommended for this task¹⁴. Oral health is important for quality of life not only aesthetically but also to prevent other bad alignments such as denture stomatitis and links with pulmonary infection^{13,19}. A good oral hygiene gives protection against microbial infections and environmental threats. According to the World Health Organization due to the increase in geriatric population worldwide it is estimated that the number of edentulous patients will also increase⁴.

The continued use and durability of dentures contributes to the accumulation of biofilm (dental plaque), staining and in extreme cases calculus^{14,20}. The mouth environment, presents ideal conditions for the growth of biofilms, in the form of moist conditions, saliva and a wide range of microorganisms²¹.

Amongst the plethora of microbes comprising the oral microbiota, there is a yeast, *Candida albicans*, that in an opportunist pathogen and is one of the relevant factors of bad alignments such as denture stomatitis and some systemic infections like invasive candidiasis^{22,23}. Poor denture hygiene will give rise to a reservoir for such dangerous organisms leading to recurrent infections¹⁹. Therefore to maintain a clean denture the ideal cleaning product needs to possess good bleaching capabilities and be an excellent antimicrobial agent^{20,24}.

1.2 Denture Materials and Cleaners

Currently there are two main materials used to manufacture removable dentures resins and metal alloy frameworks²⁵. The first ones are regularly made of polyacrylic resins, because of its low cost and easy manipulation in the manufacturing process allowing to make a fully customisable product^{26,27}. A combination of polymethyl methacrylate (PMMA), polyethyl methacrylate (PEMA) (Figure 1) with the addition copolymers plus a peroxide initiator and a pigment are the most used materials to manufacture dentures²⁷. The ratios for each composite usually vary between denture manufacturers and the final properties of dentures such as tensile strength, bend strength, water sorption and solubility are influenced by different compositions²⁸.

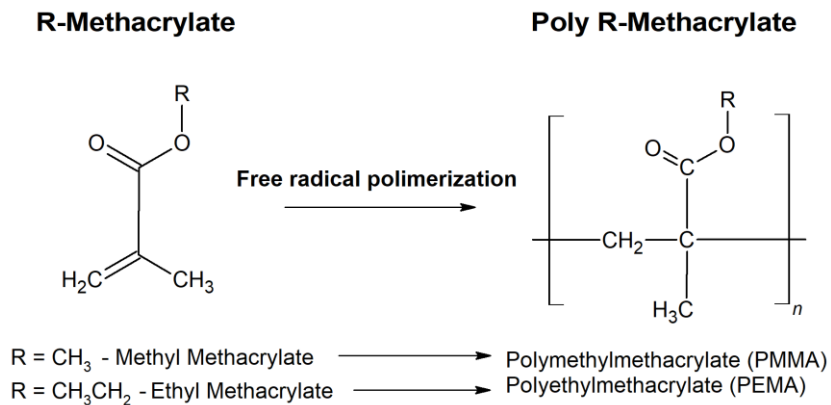


Figure 1 - Polymers used in the manufacturing of dentures. On the left side are shown the monomers, (methyl methacrylate and ethyl methacrylate depending if R is an ethyl or methyl group) and the resulting polymer single unit.

There are also the metal-based frameworks that are made of cobalt-chromium or titanium alloys and use acrylic bound to the metal to make the teeth and gums. In comparison to the resin made dentures the metal alloys tend to be lighter, with thinner sections and offer a better fit²⁵. Both frameworks are then complemented with teeth that can be also made of resin or ceramic.

The dentures can be partial or full dentures, the full dentures rest on the gums of the patient and can be kept in place using some adhesives if necessary. The partial dentures are used by patients that still conserve some teeth that are used to hold the denture in place.

The toughness of the material is an important factor in the development of denture cleansers. The exact shape of the denture must be maintained to ensure the comfort and functionality of the wearer, since they are custom-made for each person. The loss of coloration can also occur from the cleaning of dentures therefore it is important to remove stains but the original colour of the denture should not be affected^{27,29}. An ideal denture cleaner must be effective when dealing with stains without being too severe with the denture material and must have a wide range of antimicrobial activity^{20,30-32}.

Polident (Figure 2) is a commercially available product in the form of effervescent dissolving tablets to clean and disinfect dentures, a simple solution that only requires the denture to soak in it overnight. The system used in Polident is based on the generation of ROS. Peracetic acid is generated in situ by the reaction between tetraacetythylenediamine (TAED) (acyl group donor) and hydrogen peroxide³³. Table 2 shows the Polident ingredients and their contribution to the final product and concentration in the tablet.



Figure 2 – Polident tablet

Table 2 - Polident formulation and function of each ingredient adapted from the manufacturing instructions provided by GSK³⁴.

| Ingredient | Function | Weight (g/tab) |
|--|----------------------------|-----------------------|
| Citric acid | Effervescence | 0.50-0.60 |
| FD&C blue #1 Al. lake FD&C blue #2 FD&C yellow #5 FD&C yellow #5 Al. lake | Colorants | 0.002-0.003 |
| Flavour Triplemint | Flavourant | 0.02-0.03 |
| polyethylene glycol 8000 | Lubricant | 0.06-0.07 |
| potassium monopersulfate | Cleaning and Antibacterial | 0.25-0.35 |
| sodium benzoate | Lubricant | 0.05-0.08 |
| sodium bicarbonate | Effervescence | 1.00-1.10 |
| sodium carbonate | Stability agent | 0.24-0.27 |
| sodium lauryl sulfoacetate | Detergent for cleaning | 0.03-0.05 |
| sodium percarbonate | Cleaning and Antibacterial | 0.20-0.30 |
| <u>tetraacetythylenediamine</u> <u>(TAED)</u> | Antimicrobial | 0.08-0.09 |
| VP/VA copolymer (N-vinyl-2-pyrrolidone and vinyl acetate (60-40)) | Binder | 0.03-0.04 |
| Total | | 2.6-2.9 |

Sodium percarbonate is environmentally friendly and is easy to manufacture and contributes with H₂O₂ to the cleaning solution (NaHCO₃·1.5 H₂O₂) and TAED on other end will contribute with acyl groups essential for the generation of peracetic acid, a powerful disinfectant and cleaning agent^{31,35-38}.

Despite the corrosive effects of alkaline peroxide solutions several studies indicate that the damage caused by such solutions is not enough to be considered a problem if the products are used under the recommended conditions³⁹⁻⁴¹, taking into account that a surface roughness of 0.2 µm is needed for optimal microorganisms adhesion and subsequent biofilm growth⁴².

1.3 Bleaching

Humanity has been using bleaching reactions for a long time. It was originally used in the textile industry, but nowadays is employed in a wide range of processes in industry, household, cosmetics with the purpose of removing stains, to bleach materials and whitening⁴³⁻⁴⁶.

The mechanisms of bleaching process are rooted on the oxidation or reduction of the chromophores within the stain/impurity molecules, the reduction of the chromophore-bearing molecule can alter its optical properties and or increase its solubility and eventually remove it from the material to be bleached⁴⁷. The chromophore is the part of a molecule that gives rise to its colour when excited by visible light. In short, the difference between the excited and ground state energy of electrons in molecular orbitals within the chromophore must fall within the range of visible light to be seen⁴⁸. Particularly double and triple bonds and delocalised electronic systems, such electrons can absorb electromagnetic radiation and emit light. Therefore, oxidation of such bonds can affect the interaction between light and the electrons thus rendering the chromophore colourless, with no radiation in the visible range being emitted or absorbed⁴⁹.

In History the first bleaching processes relied on the use of sunlight and water to bleach textiles. The Sun as a source of UV light, high energy radiation like UV can break chemical bonds, therefore by exposing the chromophore to UV can destroy its bonds resulting in the inactivation of the its chromophore's visual properties. It is also important to consider that UV light can contribute to the production of reactive oxygen species by breaking the bonds of the water molecule creating hydroxyl radical ($\cdot\text{OH}$)⁵⁰, this molecule is extremely reactive towards organic compounds due to its high oxidative ability^{48,50,51}. The use of soap has been documented by numerous ancient civilizations more than 4 millennia ago⁵² but it was the discovery of chlorine, in the eighteenth century, that further developed the bleaching industry⁵³. Chlorine based compounds such as sodium hypochlorite, calcium hypochlorite, known as bleaches, also inactivate chromophores by oxidation.

This is normally achieved by generation of reactive oxygen species (ROS) such as the hydroxyl radical and singlet oxygen within the washing solution⁵³⁻⁵⁵.

Chlorine-based bleaches are broadly used by many industries, chlorine dioxide is commonly employed as bleach in paper manufacturing, textile cleaning and whitening, and in water treatment plants to clean and disinfect. Despite the effectiveness and low cost of chlorine-based bleaching many fears arise from the toxicity of chlorine compounds due to its strong oxidising capabilities⁵⁵. Typically, in household products the active ingredient concentrations are kept at minimum effective level (3 - 5% v/v), higher concentrations may lead to complications such as skin injuries by touch, and release of toxic vapours. Chlorine vapours also cause headaches, irritation of the throat, nausea and vomiting^{55,56} as a matter of fact is also used as a chemical weapon since the first world war⁵⁷.

These undesirable side effects to humans and animals coupled with the environmental damage caused by bleach to aquatic life contributed to the development of safer bleaching systems that rely on the oxidative power of hydrogen peroxide and reactive oxygen species. One of the advantages of using hydrogen peroxide resides in the fact that the final products of its decomposition are oxygen and water, presenting it as a much safer and environmentally friendly alternative^{36,43,58}.

1.4 Hydrogen peroxide and bleaching

Hydrogen peroxide (H_2O_2) belongs to the reactive oxygen species (ROS) family its degradation results in molecular oxygen as final products but is capable of generating other powerful ROS during its decomposition process. At basic pH this process starts with the dissociation of H_2O_2 into a more reactive form, known as perhydroxyl anion (HOO^-)^{43,45,47}.

Reactive oxygen species is a broad designation used to distinguish a variety of reactive molecules and free radicals created by incomplete oxygen

reduction. Oxygen is a stable di-radical, meaning that it has two unpaired electrons in separate valence orbitals. By gaining electrons (reduction) gives rise to active molecules, extremely reactive with high oxidation potential⁵⁹.

The first reduction of molecular oxygen produces superoxide anion ($\cdot\text{O}_2^-$) a further electron will originate hydrogen peroxide (H_2O_2) that when further reduced will give rise to hydroxyl radical ($\cdot\text{OH}$)⁶⁰. Hydroxyl radical is a strong electrophile that can attack virtually any molecule in its path capable of generating a downstream of free radicals^{61,62}. Homolytic cleavage of the peroxide bond (O-O) by UV light or metal ion oxidation can also generate hydroxyl radical^{44,61}.

Hydrogen peroxide bleaching is widely used by the textile and dentistry sectors as well but also plays a significant role in paper pulp bleaching. The dissociation into perhydroxyl anion (HOO^-) has been shown to be relevant for bleaching, taking into account that hydrogen peroxide is a very weak acid with $K_a 1.78 \times 10^{-12} \text{ M}$ ^{44,47}.

The widely accepted mechanism for hydrogen peroxide bleaching begins with dissociation into perhydroxyl ion that will then react with a hydrogen peroxide molecule to generate hydroxyl radical and the superoxide anion (Figure 3)^{47,63}.

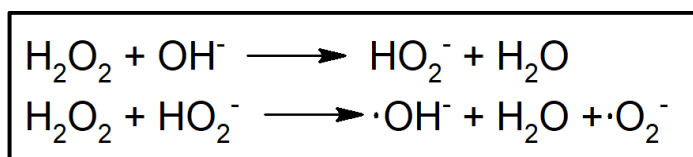


Figure 3 - Hydrogen peroxide decomposition in alkaline pH^{47,63,64}.

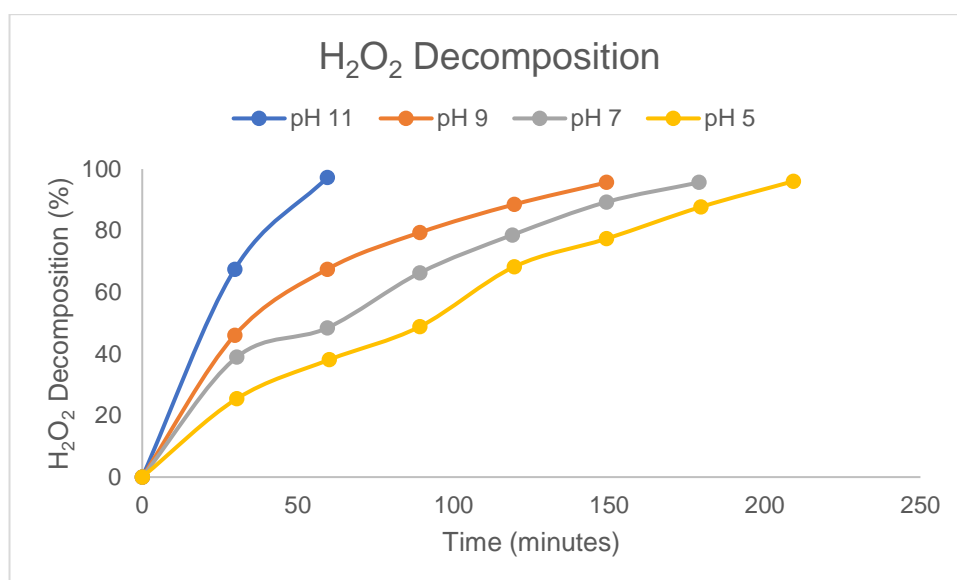


Figure 4 – pH effect on H₂O₂ decomposition, figure adapted from Abdel-Halim & Al-Deyab (2013)⁶⁵.

Figure 4 presents the findings from an investigation to observe the effectiveness of activated hydrogen peroxide bleaching. The study concluded that the decomposition of H₂O₂, was greatly favoured at basic pH and the higher the better. Other points addressed by their research were the damage to the fabric and the whiteness index achieved by the bleaching process using H₂O₂⁶⁵.

The lower pH increased weight loss and damage to the fabric (cotton), and also presented lower whiteness index, used to measure bleaching efficiency, but in comparison to higher pH, was more gentle with the material while achieving better bleaching efficiency⁶⁵.

The dissociation rate of H₂O₂ is affected by temperature, increasing 2.3 times for each 10 °C increase in temperature⁵². To achieve better results paper and textile industries use temperatures close to 100 °C and a pH between 10 and 12^{35,47}.

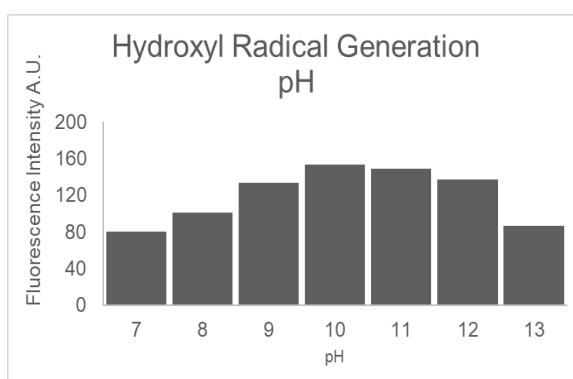


Figure 5 – Hydroxyl radical generation at different pH adapted from Si et al. (2014)⁶⁶

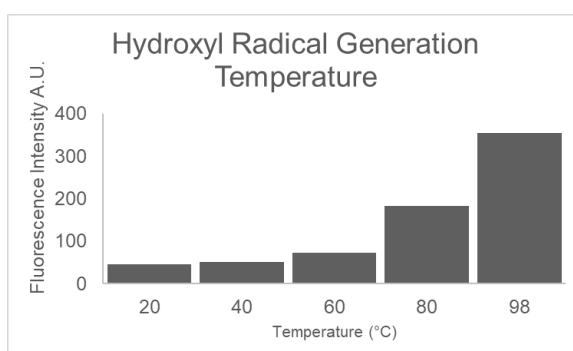


Figure 6 – Effect of the temperature on Hydroxyl radical generation at pH 10 after 60 minutes adapted from Si et al. (2014)⁶⁶

Using a fluorometric probe, benzenepentacarboxylic acid, Si and co-workers⁶⁶ quantified the generation of hydroxyl radical resulting from perhydroxyl anion reaction (Figure 5 and 6). Their method proved successful to quantify the reaction and confirmed that by increasing the temperature, the quantity of hydroxyl radical in solution was also increased. The same work also showed that the bleaching effectiveness increased with the concentration of hydroxyl radical and the optimal pH for hydroxyl radical generation was between 10 and 11, which is also the optimal pH for dissociation of H₂O₂ into the perhydroxyl anion⁶⁶.

Those extreme conditions used for an effective bleaching with H₂O₂ are not suitable for most of the materials that need bleaching and to address such problems new ways to harness the H₂O₂ power at lower temperatures and pH were devised^{47,65}.

Taking into account that H₂O₂ bleaching is a kinetic process one way to increase the effectiveness of the process will obviously be increase exposure time and or increase its concentration in solution⁴⁵.

Despite the fact that H₂O₂ is environmentally safe its use can also bring some problems due to the oxidative nature its products can present; it can generate free radicals and oxygen. H₂O₂ is stable in the absence of catalysts such as metal ions, UV light or heat. On its own it is not flammable, but the oxygen generated during its decomposition can present a problem in the form of a fire initiator. During prolonged or inadequate storage oxygen can be released partly due to a disproportionation reaction and this gas release can increase the pressure within the container making it explode and release oxygen⁵². As consequence, in liquid formulations, stabilizers and acid buffers must be employed, another option is to use persalts with peroxy groups that are much more stable and can be used in a solid formulation^{45,52,58}.

Among peracids the most widely used are sodium perborate and sodium percarbonate. Sodium perborate that normally appear in two forms sodium perborate monohydrate (NaBO₃*H₂O) and sodium perborate tetrahydrate (NaBO₃*4H₂O) they are true peroxides and possess a B-O-O-B group^{36,67}. Sodium percarbonate, (Na₂CO₃*1.5H₂O₂) which has a solvated hydrogen peroxide group that when dissolved dissociates in carbonate and H₂O₂. Environmental concerns related to the release of boron and perborates to the environment have favoured sodium percarbonate use⁶⁸. Apart from the environmental concerns sodium percarbonate also presents the advantage of contributing to an increase in the pH that favours the H₂O₂ dissociation into more reactive forms^{52,68}.

Figure 7 shows sodium perborate and sodium percarbonate chemical structures^{69,70}.

An important feature to consider when using persalts, is the content of active oxygen. The active oxygen content relates the total mass of oxygen and the total mass of the compound given by the following formula⁵²:

$$\text{Active oxygen \%} = \frac{\text{No. of oxygen atoms in peroxide groups} \times 16}{(\text{Molecular weight of the compound})} \times 100$$

Equation 1 – Active oxygen content determination⁵².

Table 3 – Active oxygen content of H₂O₂, sodium perborate and sodium percarbonate⁶⁸

| | Hydrogen Peroxide | Sodium Perborate Tetrahydrate | Sodium Perborate Monohydrate | Sodium Percarbonate |
|--------------------------|-------------------|-------------------------------|------------------------------|---------------------|
| Active Oxygen (%) | 47% | >10 | >15 | 13 |
| Solubility (water) (g/L) | miscible | 23 | 15 | 140-150 |

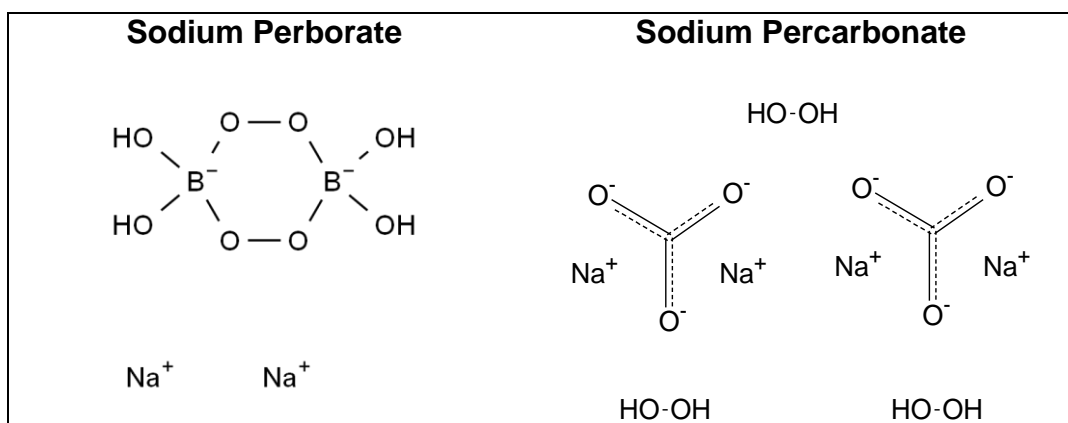


Figure 7 – Sodium perborate salt (left) and sodium percarbonate (right).

Despite having less active oxygen than H₂O₂ (table 3) peroxide donors present an excellent alternative since they can eliminate some stability and shelf life limitations but did not affect the need of high temperature and pH. The development of H₂O₂ activators come to rescue the bleaching process from the high temperature and pH dependence^{37,47,67,71}.

1.5 Hydrogen Peroxide Activators

Transition metals like iron, copper and manganese and their complexes at lower oxidation states, at low concentrations and acidic pH, behave as catalysts for the homolytic cleavage of hydrogen peroxide into hydroxyl radical ($\cdot\text{OH}$) this process is limited by the concentration of the metal, if their concentration is too high the metal itself can act as a scavenger for the reactive oxygen species or react with itself and have a lower oxidation potential this reaction is known as the Fenton reaction^{45,47,65,72,73} (Figure 8). This process was first described by Fenton in 1894 where tartaric acid was oxidised in the presence of iron and H_2O_2 ⁷⁴ and was later identified as an essential process taking place within living organisms to generate free radical that can be used as signalling molecules, effective to kill pathogens by white blood cells⁶⁰. Due to its effectiveness in generating powerful ROS the Fenton reaction is also used in waste water treatment plants to purify and clean the water⁷⁵.

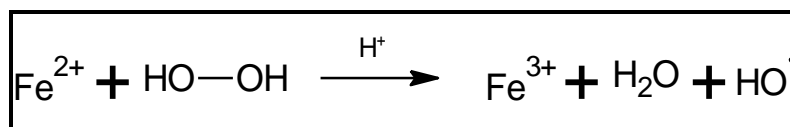


Figure 8 - Fenton Reaction. Ferrous ion reacts with hydrogen peroxide in acid environment to originate Ferric ion, a water molecule and hydroxyl radical⁷⁵.

The ideal solution to release the power of H_2O_2 appeared in the form of peracids. This is achieved by developing hydrogen peroxide activators that contain acyl groups ($\text{C}=\text{O}$) attached to stable leaving groups, generally imides or esters⁴⁵ also known as peracid precursors. Those precursors in the presence of perhydroxyl anion release the peracid corresponding to the acyl group targeted^{43,67} see Figure 9. The resulting peracid is responsible for the bleaching activity and the acyl group can be modified, by adding properties to target specific types of stain. The leaving group plays a role in the solubility of the compound that can affect its reaction rate and stability^{36,45,52}.

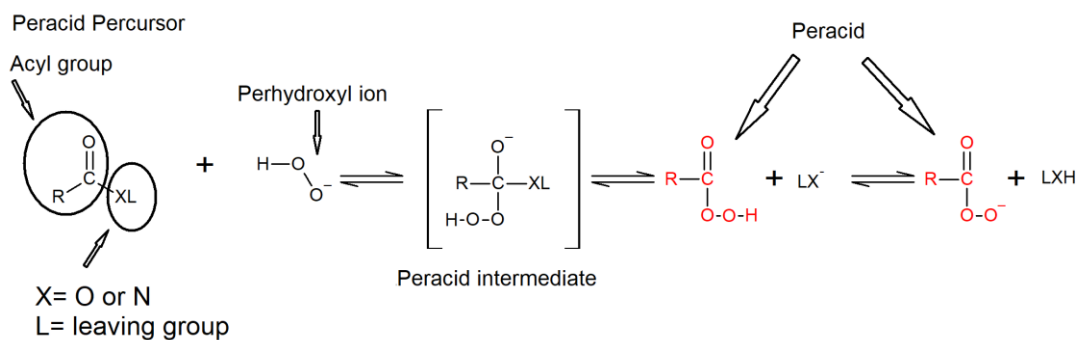


Figure 9 – Schematic of formation of peracids.

The accepted reaction mechanism of peroxide activators suggests that the perhydroxyl anion, resulting from the dissociation of H_2O_2 ^{76,77}, targets the acyl group forming a peracid intermediate that will then terminate with the release of the leaving group. The removal of perhydroxyl ion will contribute to the increase of the perhydrolysis of H_2O_2 reaction effectively reducing the pH requirements when using of H_2O_2 alone. The newly formed peracid also has the capability of reacting with itself and generate further ROS and free radicals with both being effective in the inactivation of chromophores by oxidation as hydrogen peroxide³⁷.

The properties and kinetics of commonly used hydrogen peroxide activators have been widely studied^{37,43,67,78–81} with detailed explanations regarding the ROS generated and its activation conditions and suitability for the final use applications. The mechanisms behind peracids (Figure 10) produced by some H_2O_2 activators including differences between anionic and cationic peracids that can be used to target specific features of the material to be bleached⁴³ are well known as well further understanding into the conditions that need to be met by domestic laundry products that use oxygen bleaching⁴⁵. When choosing an activator, apart from being financially viable and having low toxicity, they must be compatible with the final product formulation, be reactive under the required bleaching conditions, that are limited by the resistance of the material to be bleached or the bleaching vessel/equipment^{45,67}.

At lower temperatures (40-60 °C) peracids are much more effective than H_2O_2 at bleaching thanks to their higher oxidising potentials⁵⁸.

| Structure | Name |
|---|---|
| $\text{R}-\text{O}-\text{O}-\text{H}$ | Alkyl or aryl hydroperoxydes |
| $\text{R}-\text{O}-\text{O}-\text{R}'$ | Dialkyl or diaryl peroxides |
| $\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-\text{O}-\text{H}$ | Alkyl or aryl per- or peroxy-carboxylic acids |
| $\text{R}-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-\text{O}-\text{R}'$ | Alkyl or aryl per- or peroxy-esters |
| $\text{R}-\overset{\text{O}}{\parallel}{\text{S}}(\text{O})_2-\text{O}-\text{O}-\text{H}$ | Alkyl or aryl persulphonic acids |

Figure 10 – Example of peracid compounds⁸².

When dissolved H_2O_2 activators can react in three manners: (i) - hydrolysis in the presence of $\text{H}_2\text{O}/\text{OH}^-$ that generates the analogue carboxylic acid, (ii) - perhydrolysis by $\text{H}_2\text{O}_2/\text{HOO}^-$ producing the desired peracid and (iii) - diacylperoxyde (DAP) formation that occurs upon reaction between the recently formed peracid anion and the acyl donor moiety, as seen in Figure 11⁵⁸.

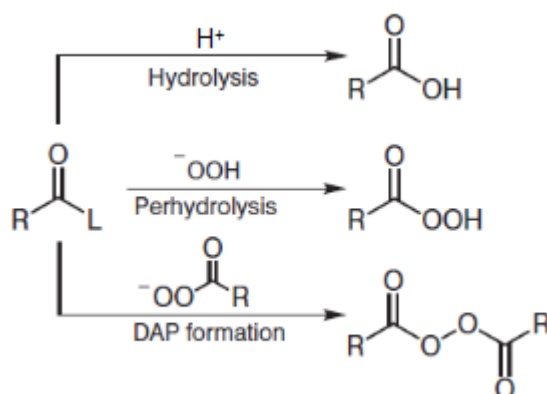


Figure 11 – H_2O_2 activator decomposition⁵⁸.

The pH of the solution plays a major role in the selection of the decomposition route and final products. Higher pH tends to select the DAP formation while an acidic pH will favour the hydrolysis. The perhydrolysis reaction normally takes place at a pH defined by the stability of the peracid.

From all the decomposition routes the only one that does not contribute to the generation of ROS is the hydrolysis at acidic pH^{45,58}.

Nowadays the main bleach activators used in household products are nonanoyloxybenzene (NOBS) and tetraacetythylenediamine (TAED)^{52,72}. Figure 12 presents three bleach activators TAED, NOBS and a novel cationic bleach activator (N-[4-(triethylammoniomethyl) benzoyl]-caprolactam chloride that is used to target anionic fibres of a textile material.

Since this study is directed towards the peracetic acid and singlet oxygen generation the TAED/H₂O₂ system will be the focus of this study. TAED satisfies most of the required criteria for an excellent bleach activator. TAED/H₂O₂ is widely used in paper and textile industry including applications in household laundry products, among other cleaning and sanitizing products such as denture cleansers. Preferentially, TAED reacts with the perhydroxyl ion leading to the generation of peracetic acid (PAA), that is a stable peracid with the advantage of not forming diacyl peroxides^{43,45,58,71}.

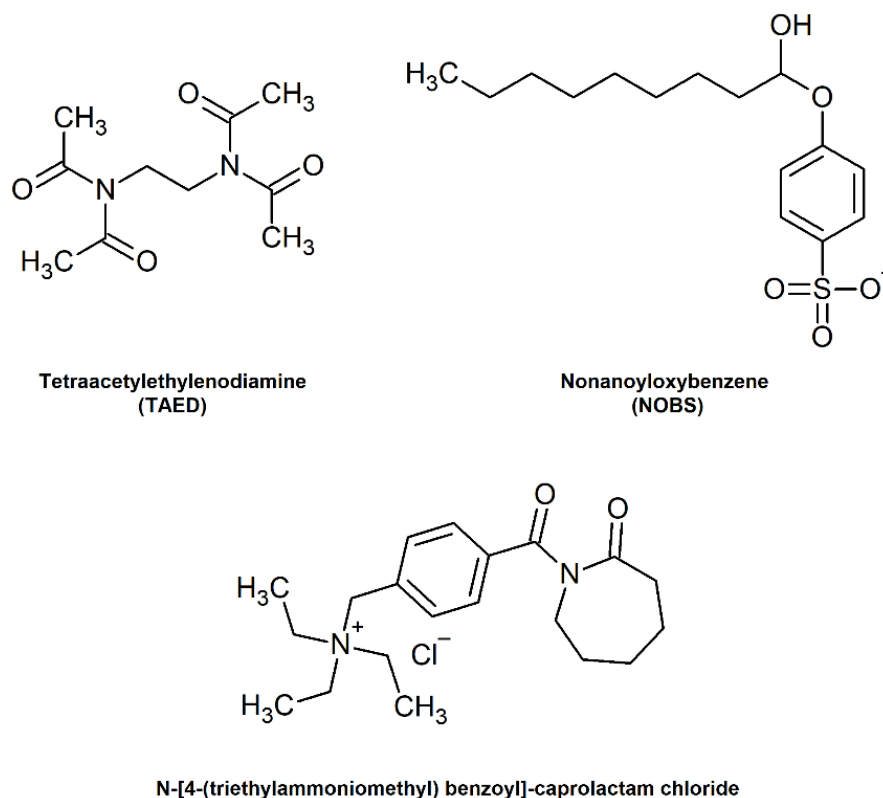


Figure 12 – Example of bleach activators^{35,38}.

Despite TAED containing four acyl moieties (R-C=O) only two are released originating two molecules of peracetic acid and one of diacetythylenediamine (DAED) Figures 13 and 14. The high pKa of the conjugate acid conversion of an amide to an amine impedes this process⁶⁷.

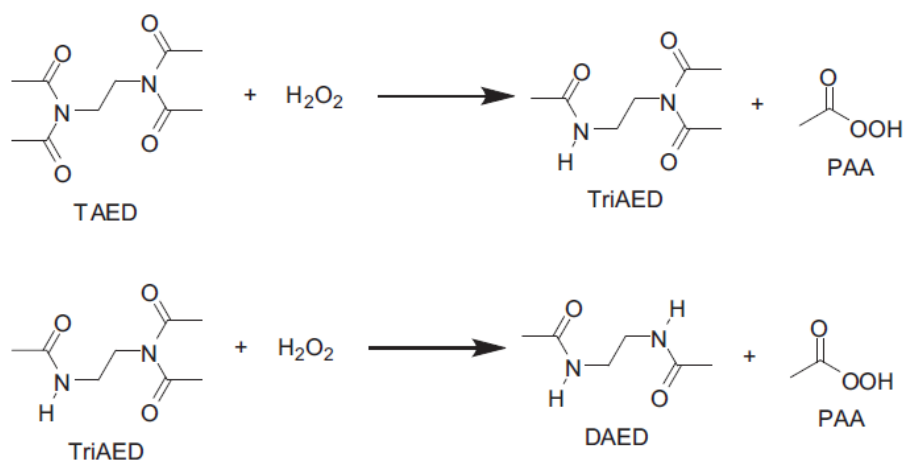


Figure 13 - Perhydrolysis of TAED^{35,38}

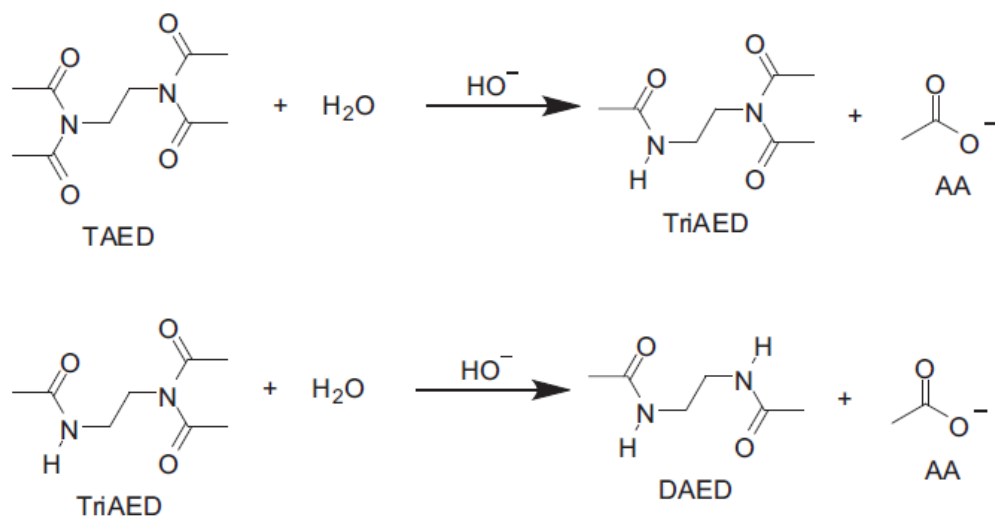


Figure 14 – Alkaline hydrolysis of TAED³⁵.

As stated before peracid formation is pH dependant, a pH between 8-12 promotes the alkaline hydrolysis of TAED, producing acetic acid and DAED (Figure 14) while lower pH (3-7) the perhydrolysis is the main formation route producing the desired product, the peracetic acid³⁸.

1.6 Peracetic acid as alternative to H_2O_2

Peracetic acid (PAA) is known to be a very strong oxidant with disinfectant properties with a broad range of bactericidal, antimicrobial, fungicidal, anti-viral, sporocidal and bleaching properties both at low concentrations and low temperatures. Due to its effectiveness PAA has been employed in wide range of uses and products applications for household, hospitals and various industries^{30-32,82}.

The conjugate peroxide radical is very energetic and thermodynamically unstable under the optimal conditions. PAA is water soluble and as for other peracids it is extremely reactive towards oxidised compounds generating large amounts of heat in the process that can be sufficient to cause ignition³¹. In comparison, PAA has a stronger oxidation potential than chlorine and chlorine dioxide. Regarding its toxicity PAA shows

very low toxicity but it can be irritating to eyes, skin and mucous membranes, and is capable of inducing burns at concentrations higher than 40%⁸².

Table 4 - Oxidizing power of common disinfectants^{55,83}.

| Oxidant | Oxidation Potential, V |
|-------------------|------------------------|
| Peracetic Acid | 1.81 |
| Hydrogen Peroxide | 1.80 |
| Chlorine Dioxide | 1.57 |
| Chlorine | 1.36 |

It is known that PAA in solution, as for other peracids, decomposes through three different routes as shown in Figure 15 that are: (i) spontaneous decomposition, (ii) hydrolysis and (iii) metal ion-catalysed reactions. The optimal pH range that produces singlet oxygen lies between 5.5 and 8.2. Within this range the major decomposition route is the spontaneous decomposition. Higher pH favours metal decomposition if metals are present in solution and the last decomposition route the hydrolysis is present at lower pH below 5 but also at a pH around 10.5 producing of acetic acid and H₂O₂^{84,85}. An increase in temperature will be advantageous for the reaction to take place but too high temperatures can lead to the PAA being decomposed before reacting with the stain chromophores⁷¹.

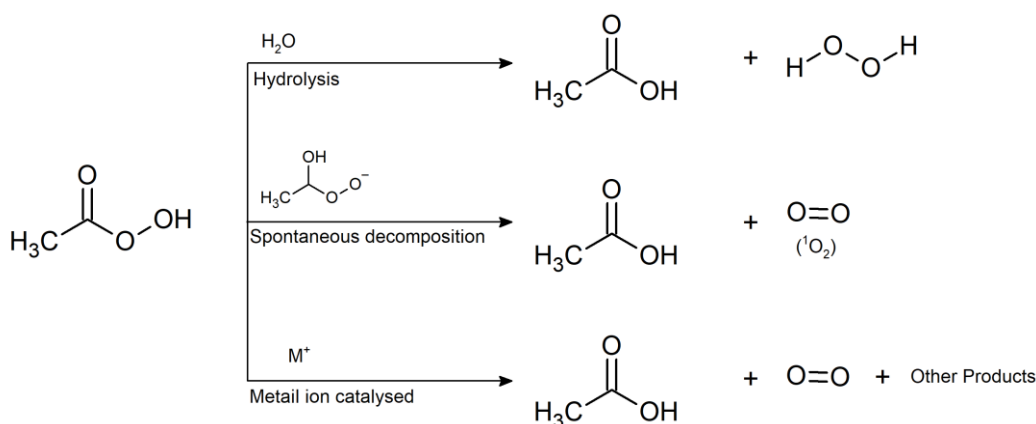


Figure 15 – Peracetic acid decomposition routes⁸⁵.

There are commercial solutions of concentrated peracetic acid in the market, they are made as a mixture of peracetic acid, acetic acid and H₂O₂ in equilibrium with a content of peracetic acid ranging from 5% to 42%. Peracetic acid in storage is also liable to undergo decomposition a factor that

impairs its shelf life, therefore stabilisers need to be in place to prevent this from happening⁸². PAA is much safer and easy to handle and delivers superior cleaning and bleaching effects than H₂O₂ at lower temperatures.

1.7 The TAED/H₂O₂ system

As previously elucidated in Figure 13, the peracid generated by the reaction between tetraacetythylenediamine (TAED) and sodium percarbonate (NaPC) is the peracetic acid (PAA). With TAED acting as the acyl group donor and H₂O₂ supplied by the dissociation of sodium percarbonate, with the formula 2NaHCO₃ · 3H₂O₂. Sodium percarbonate is environmentally safe and is easy to produce. Its chemical structure and production optimization, including storage shelf-life have been widely researched^{69,70}. The following Figure 16 shows the relevant processes taking place leading to the generation of singlet oxygen through the reaction between TAED and NaPC. First TAED and NaPC will dissolve in solution and subsequently NaPC will dissociate into its constituent's sodium carbonate and hydrogen peroxide as shown in reaction 1. This is followed by the formation of perhydroxyl anion (HOO⁻) due to dissociation of the H₂O₂ in aqueous solution (2). Then this much more reactive form of H₂O₂ will target one of the acyl groups of the TAED molecule leaving us with DAED, Figure 17 b), and peracetic (3), Figure 17 a), finally at the optimal pH two molecules of PAA, can further react with each other and produce the much reactive singlet oxygen (4).

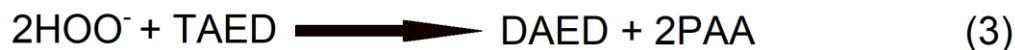
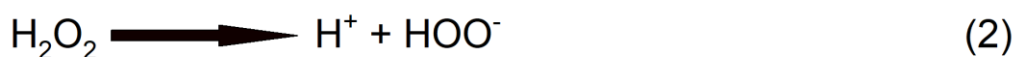
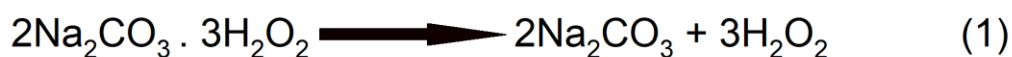


Figure 16 – Peracetic acid and singlet oxygen generation by reaction between TAED and NaPC^{35,38,86}. (1) NaPC decomposition, (2) hydrogen peroxide dissociation, (3) diacylation of TAED, (4) peracetic acid spontaneous decomposition.

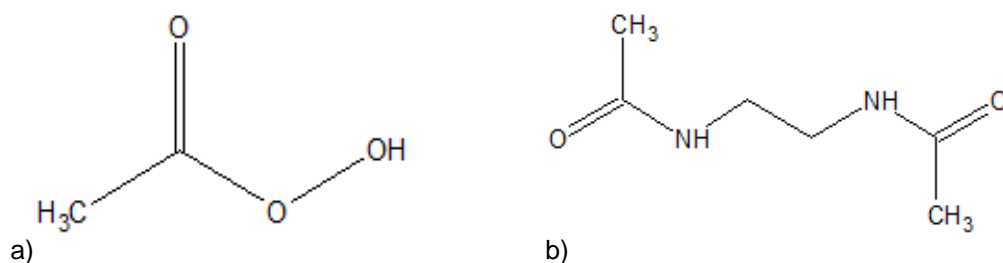


Figure 17 – TAED / H₂O₂ Reaction Products. a) peracetic acid, b) diacetylenediamine (DAED).

Research conducted by Si *et al.* (2014)⁸⁷, showed that the amount of hydroxyl radical generated by the TAED and H₂O₂ was greatly increased when compared to H₂O₂ used on its own. By using an hydroxyl radical (•OH) scavenger the bleaching effectiveness was reduced, elucidating that the production HO• generation also has a role in bleaching⁸⁷. When the mechanisms and the preparation of peracetic acid and the kinetics of its decomposition in the liquid phase at different temperatures were investigated it was shown that they followed a first order reaction mechanism for the hydrolysis and spontaneous decomposition while presented a second order mechanism for the metal ion catalysed decomposition⁸⁰. Temperatures below 54 °C favoured hydrolysis decomposition route and a decrease in pH also reduced the spontaneous decomposition route⁸⁵.

At a pH of 8.2, the major decomposition route is spontaneous decomposition but the other decomposition routes, metal catalysis and

hydrolysis were also present. Laundry systems rely on the use of metal scavengers to minimize the metal-ion catalysis effects⁸⁰.

Bleaching studies using peracetic acid have also proved that at low temperatures it is more effective than H_2O_2 ^{38,82,87}. An experiment using fluorescence to detect the generation hydroxyl radical demonstrated that H_2O_2 dissociation rate, on its own, is highly favoured by high pH and high temperatures that increased the amount of perhydroxyl ion in solution when compared to its bleaching effectiveness against the activators TAED and N-[4-(triethylammoniomethyl)-benzoyl]-caprolactam chloride (TBCC). At 70°C the dissociation of a 5g/L H_2O_2 solution was 43.67%. Using TAED as activator increased the decomposition to 67.56%. and TBCC increased the decomposition to 76.33%⁸⁷.

The whiteness index is a quantification method used to determine the efficacy of textile bleaching agents. This is a comparative method where dirty fabric is challenged by the bleaching agent and compared against a control sample of clean material. After wash the materials are dried and a colorimeter or a spectrophotometer are used to measure the light reflectivity, the whiter the material the more light will be reflected thus giving us a measure to quantify bleaching efficacy. These methods are usually standardized and employed by textile manufacturers following guidelines established by trade regulators like the American Association of Textile Chemists and Colorists and homologue organizations⁸⁸. Whiteness index studies have shown that the TAED/ H_2O_2 system at low temperatures is more effective than H_2O_2 at removing stains^{35,43}.

Peracetic acid generated by the TAED/ H_2O_2 system presented an increase in whiteness index in the pH range from 4 to 8, and at a pH of 9 or higher the whiteness index decreased³⁸, not surprisingly, this corresponds to the ideal pH for spontaneous decomposition of peracetic acid⁸⁰. High pH favour the hydrolysis decomposition route of peracetic acid and generates H_2O_2 but the effects of alkaline decomposition of TAED become too great to be neglected and will affect the generation of peracetic acid due to depletion of TAED^{38,80,82}.

Other studies focused on the effects of the concentration of H₂O₂ within the system. At a pH of 7 an increase in the H₂O₂ concentration greatly enhanced the bleaching activity of the TAED system. At pH 10 there was an increase in the whiteness index obtained by the TAED/NaPC system, but to a lesser extent than for H₂O₂ at pH7. When the pH was raised to 12, there was no difference between the whiteness indexes of both TAED/NaPC and H₂O₂ solutions. In any of the conditions, H₂O₂ in excess did not result in a significant increase in whiteness of the fabric showing that the reaction between TAED and H₂O₂ is the major effector in bleaching efficacy³⁸.

As previously elucidated by other works a pH of 12 will not favour the optimal TAED degradation nor the subsequent spontaneous decomposition of peracetic acid but as a matter of fact this level of pH is ideal for H₂O₂ bleaching⁴⁵ therefore, the bleaching obtained at this pH originates from the oxidative properties of H₂O₂ on its own^{35,38,80}.

Since the main reactions taking place are pH dependant, it must be considered that the generation of acetic acid as final product will contribute for a decrease in the pH of the system making essential to consider the use of a buffered system to increase the effectiveness of the product^{32,82,85,87}.

Overall studies using peracetic acid in fibre bleaching clarified its potential and capabilities and elucidated its main advantages as its overall cost in comparison with other bleaching systems used by the industry without disregard of the safety issues that can be avoided by using an *in situ* method for the generation of the main bleaching agent and savings prompted by bleaching at low temperatures. It is also important to take into consideration that one of the major limitations of this system is the low solubility of TAED 1.2 g/L at 20°C^{43,52,58,82}, by using this method the reagents costs are not greatly reduced but the bleaching quality is improved and the damage to the materials and environment is reduced³⁵. All together those qualities made the TAED/H₂O₂ system very attractive, as consequence, TAED is the main bleach activator used in Europe for household textile washing.

1.8 Antimicrobial Properties of Reactive Oxygen Species

Higher living organisms rely on reactive oxygen species such as H_2O_2 and some nitrogen species to regulate host defence, as a chemical messenger to mediate inflammation and actively participate in the killing of invading pathogens^{89,90}. Studies monitoring the oxygen consumption of leucocytes have shown an increase in oxygen consumption when in the presence of bacteria, this phenomena was subsequently credited to the generation of the ROS used to kill pathogens⁹¹.

Reactive oxygen species are more effective against Gram-positive bacteria than Gram-negative bacteria but that can also be affected by the presence of ROS scavengers that can contribute to an increase in the tolerance towards oxidative damage by such organisms^{89,92}.

In the case of oxygen activation by phagocytes this has as final product superoxide ($\text{O}_2^{\cdot-}$) that will then contribute in the production of more ROS such as H_2O_2 , $\cdot\text{OH}$, $^1\text{O}_2$. These molecules can then target and originate other antimicrobial molecules by radical chain reaction propagation and/or serve as signal to other cells. ROS molecules can target various structures within the microbial cell, such as thiols, metal centres, protein tyrosines, DNA and lipids^{59,93} and damage to these structures will eventually lead to cell death. The low specificity and the wide range of targets combined with the low toxicity towards the environment make ROS an excellent antimicrobial agent. As a matter of fact, there are plenty of products using ROS in the market in cleaning and disinfection applications that make the most of the excellent antimicrobial properties^{59,89}.

Studies with *Escherichia coli* and H_2O_2 showed that at low concentrations of H_2O_2 (1-3 mM) DNA damage was the main cause for cell death. While at higher concentrations the damage was more generalised, there is also evidence showing that the DNA damage was dependent on iron suggesting that, through the Fenton reaction, hydroxyl radical or other ferrous product was taking part in this mechanism^{59,90,94}.

In the same way as H₂O₂, peracetic acid (PAA) is also regarded as an excellent antimicrobial agent, it shares a similar antimicrobial activity as H₂O₂ with the advantage of requiring much lower concentrations, a solution of 0.3% PAA has the same antimicrobial activity as a solution of 10 to 30% of H₂O₂⁹². It is thought that peracetic acid inactivates bacteria in a similar fashion as H₂O₂ by targeting proteins and enzymes and increasing cell wall permeability⁸⁹.

PAA is normally sold as a mixture of peracetic acid, hydrogen peroxide and acetic acid in liquid form for disinfectant and cleaning applications and is widely used in healthcare environments and regarded by some as “the new hero in hospitals”⁹⁵. Its efficacy has also been proven in the cleaning and biofilm removal of cooling towers³⁰, treatment of household waste water for disinfection and to breakdown debris⁸³.

Another important factor is that resistance of bacteria towards peracetic acid has not yet been observed⁹⁶.

1.9 Denture Biofilms and Denture Stomatitis

Biofilms are aggregates of bacteria that live attached to a surface surrounded by a self-made sugary matrix^{6,21,97}. Biofilms can be found in every environment on our planet including natural surfaces and manmade structures. They represent a major problem in the healthcare sector because within biofilms bacteria are more resistant to antibiotics and disinfectants and if left unchecked can become a reservoir for recurring infections and outbreaks^{6,8,98,99}.

Biofilm formation start when some bacteria land on a surface and adheres to it and start producing a sugary matrix. This sugary matrix will them become the scaffold for more other bacteria to attach to it and at this stage the biofilm start maturing^{10,11} (Figure 18). Within the biofilm matrix the organisms within it can change DNA, metabolites and effectively communicate within the biofilm⁶.

The oral biofilm or dental plaque is one of the most widely studied biofilms in nature. Recent reports identified more than 700 bacterial species although not all the species identified can be cultured using standard methods, DNA based culture independent methods such as PCR and gel electrophoresis played a vital role in our understanding of the oral microbiome^{100,101}. Apart from bacteria there are also *Fungi*, *Archaea*, viruses and *Protozoa*¹⁰².

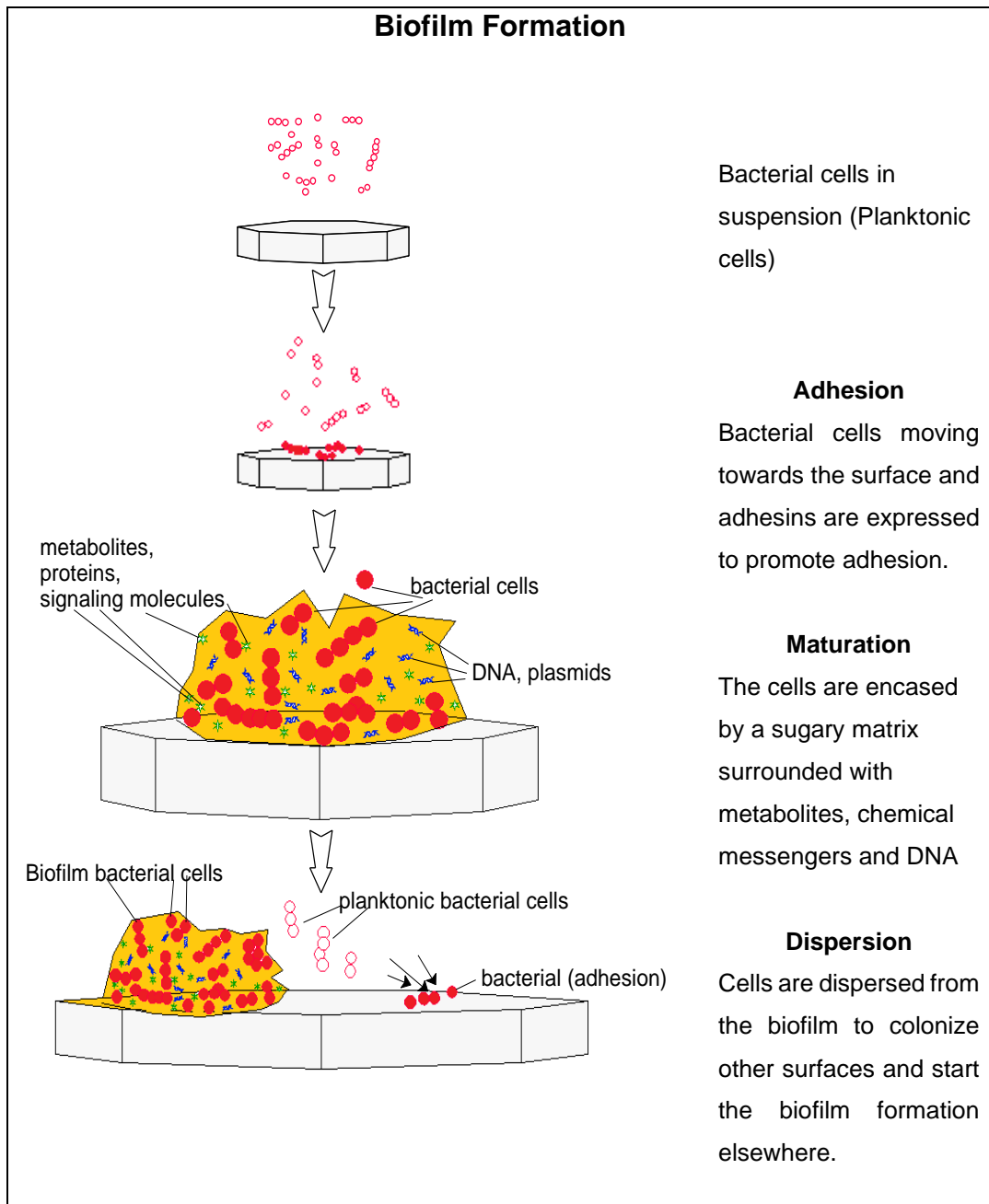


Figure 18 – Biofilm formation schematic. Biofilm formation process start with the adhesion of planktonic cells to a surface, then the cells will start to produce a sugary matrix that will act as scaffold for more cells to attach and metabolites and DNA circulate within the biofilm matrix. The last phase of biofilm is the dispersion were cells detach from the biofilm and become planktonic to colonize another region.

The dental plaque formation is not a random process, it follows well-structured mechanism, it starts when the surfaces in the mouth become coated with glycoproteins present in saliva, that will promote the adhesion of facultative anaerobes such as *Actinomyces* species and oral Streptococci¹⁰³. Those organisms are commonly referred as the early colonizers and are the ones that will produce the polysaccharide matrix for the biofilm and at a later

stage present receptors for bonding with the secondary and late colonizers.¹⁰ The process starts when the bacterial cells sense a surface and start expressing specific genes that will lead to the production of proteins (adhesins) that will help the cells to attach to structures found in the surfaces¹⁰⁴. The secondary colonizers contrary to the early colonizers require specific receptors and adhesins to attach themselves to the biofilm, hence the need for the early to colonizers to prepare the ground. Among the secondary colonizers are species like *Fusobacterium*, *Veillonella* and *Prevotella* spp.¹⁰ Subsequently the late colonizers are added to the biofilm *Eubacterium*, *Treponema* spp., *Pseudomonas gingivalis*, *Selenomonas flueggii* and *Aggregatibacter actinomycetemcomitans*^{7,103}.

Research shows that around 141 different bacterial species can be found in an individual dental plaque, representing six distinct bacterial phyla the *Firmicutes* (e.g. species of *Eubacterium*, *Gemella*, *Selenomonas*, *Streptococcus*, *Veillonella*, and related ones), the *Actinobacteria* (e.g. species of *Actinomyces*, *Atopobium*, *Rothia*, and related ones), the *Proteobacteria* (e.g. species of *Neisseria*, *Eikenella*, *Campylobacter*, and related ones), the *Bacteroidetes* (e.g., species of *Porphyromonas*, *Prevotella*, *Capnocytophaga*, and related ones), the *Fusobacteria* (e.g., species of *Fusobacterium* and *Leptotrichia*), and the TM7 phylum, for which there are no cultivable representatives¹⁰⁰.

The mouth cavity environment offers the ideal conditions for the formation of biofilms it is humid and the saliva play a significant role in the transport of nutrients and maintaining optimal pH levels plus a combination of soft and hard surfaces make it a unique environment for biofilm colonization by adding a denture we are effectively increasing the surface area of the mouth cavity that will result in more places regions for the organisms to thrive^{16,105}.

The organisms found in denture biofilms do not change significantly from the ones found in normal teeth only the proportions of some species are altered. Also after cleaning the biofilm develops quicker and in a more complex way in natural teeth than in artificial teeth¹⁰⁶. In a clinical study

Actinobacteria and Bacilli were the predominant classes found in dentures and mucosal sites of denture wearers accounting for 75.2% and 66.4% respectively while in normal dental plaque they only account for 30% of the total biomass, the same study also showed that dental plaque is more diverse than denture plaque¹⁰⁷. Other interesting factor is the increase in the fungus from the *candida spp.* *Candida albicans* and *Candida glabrata* are the most common species found and their numbers tend to increase from partial dentures to complete dentures^{105,107}.

C. albicans is closely associated with denture stomatitis^{17,107,108}, an inflammatory condition of the mucosal tissue in denture wearers predominantly localized under dentures^{19,109}. Sufferers of denture stomatitis report mucosal bleeding and swelling, bad breath, xerostomia sometimes associated with pain or a burning sensation¹⁰⁹.

Recent studies also suggested that dentures can act as reservoirs for respiratory pathogens¹¹⁰ and also there are some relevant links between oral pathogens and systemic disease^{111,112}. All these findings show to the relevance of maintaining good oral hygiene to prevent tooth loss. Denture wearers are more at risk of developing certain types of oral and systemic diseases than a non-denture wearer therefore special attention must be placed in the daily cleaning of a denture to prevent the accumulation of undesired biofilms and the onset of disease.

2. Aims and Objectives

The aim of this work was to explore and optimize the mechanisms involved in the generation of reactive oxygen species within a formulation and its effects in the killing and removal of biofilms. It was intended to do a reverse engineering task on a commercially available product and use its formulation as a scaffold to generate a simplified formulation with focus in the ROS generation. In order to answer the questions that arise from the topic the course of studies was divided into two distinct areas, chemistry and microbiology.

The TAED/H₂O₂ present in the Polident system was used and its capability to generate ROS analysed.

On the chemistry side the ¹H-NMR capabilities were explored to detect and follow the reaction between the main reagents in a commercially available denture cleanser tablet, Polident. And, to determine reaction orders and activation energies of the main reactions taking place. Arising from the degradation of TAED into DAED and subsequent conversion of peracetic acid into acetic acid.

Since ¹H-NMR cannot detect the resulting ROS, a complementary experiment using fluorometric probes dichlorodihydrofluorescein and singlet oxygen sensor green were employed in order to ascertain the generation of a further ROS and detect singlet oxygen. The initial findings were applied to develop the formulation and increase the reactive oxygen species output.

On the microbiology side the antimicrobial properties of Polident and its ingredients were studied against planktonic microorganisms and biofilms. More specifically we looked at the Kill Time of Polident and a placebo tablet (no actives) followed by the determination of the minimum inhibitory concentration and minimum bactericidal concentration of the above and as well the contribution of its components to the antimicrobial effects observed.

In the antimicrobial studies, were used microorganisms from the oral microbiota, since this is one of the most important biofilm communities present in humans and the most relevant for the product being

tested/developed. As oral microorganisms we used organisms commonly found in denture biofilms *Streptococcus mutans*, *Fusobacterium nucleatum* and *Candida albicans* and reproduce the oral environment to create the biofilm. In this section it was also essential to develop the biofilm formation methods to be employed in the study, so a part of the microbiology section was dedicated to the biofilm development studies.

3. Experimental Section

3.1 pH and NMR Studies

3.1.1 Introduction

Nuclear magnetic resonance spectroscopy (NMR) is a technique used to observe the magnetic field of nuclei. It is based on the principle that when a nuclei interacts with a magnetic field it resonates at a frequency characteristic of that specific nuclei and its surrounding environment^{113–115}

NMR spectroscopy is mainly used in research to characterize and identify novel compounds and provide structural information but also plays an increasingly significant role to detect small impurities and identify the components of a mixture by the pharmaceutical industry^{116,117}.

Any proton in different compounds will have a corresponding ¹H-NMR signal that will be proportional to the number of protons of the specific nuclei present in the sample, by measuring the area of the peak (integration) and having a reference compound in the solution we can effectively quantify the target compound in real time.

There is a linear relationship between signal intensity and number of nuclei originating the signal given by:

$$I = cS * N$$

Where *cS* is the proportionality constant given by the “spectrophotometer constant” and the sample, *I* the intensity of the signal and *N* the number of nuclei^{116,118}.

Proton NMR spectroscopy (¹H-NMR) was our main resource to identify the compounds present when the Polident tablet was dissolved. When all identifiable species were assigned to their correspondent chemical shift, the study focused in the regions containing the peaks corresponding to

peracetic acid, DAED and acetic acid, the products of the reaction between TAED and $\text{H}_2\text{O}_2/\text{OOH}$ and the subsequent degradation of peracetic acid.

^1H -NMR was chosen due to its precision in quantifying different chemical species within a solution, by relating the intensity of the signal (peak area) with the number of equivalent protons responsible for the signal. If we determine the area inside the peak that area will be proportional to the number of protons giving rise to it¹¹⁸. By applying this rule, the ingredients and products present in our sample were quantified and a kinetic study at different temperatures performed.

Since the reaction taking place is pH dependent alongside the NMR measurements the pH of the samples and individual ingredients was also measured.

3.1.2 Material and Methods

All the chemicals used in the experiment were provided by GlaxoSmithKline and were analytically pure. Sodium percarbonate; TAED - GMA grade, GSK Raw Material Code 508943 – Lot 141045570; Citric acid; Polyethylene glycol; Potassium monopersulphate (MPS); Sodium Benzoate; Sodium bicarbonate; Sodium Carbonate; Sodium lauryl sulfoacetate; Polident®3 Denture Cleanser, GlaxoSmithKline – Lot MD231513A; D_2O – Sigma Aldrich; Placebo tablet (tablet without sodium percarbonate, TAED and potassium monopersulfate).

pH meter – Five Easy™ FE20, Mettler-Toledo AG.

^1H - NMR – Bruker Avance III 400 and Bruker Avance 500 equipped with a gradient probe for ^1H and ^{13}C measurements set to perform 8 scans per sample.

Bruker Topspin 4.0.5 – was employed to analyse the NMR spectra.

3.1.2.1 Sample preparation for NMR and pH measurements

In the NMR and pH measurements, for the starting materials, a solution with approximately 15 mg/ml of the ingredient was prepared using D₂O and deionised H₂O. The tablet was dissolved as indicated in the product label, one tablet (2.7 g) in 150 ml of warm water (40 °C) adapted to 0.09 g in 5 ml in these experiments.

The mixture of the “actives” composition was 1:2 of TAED and NaPC, 0.05 mM and 0.10 mM respectively. On the initial screening the tablet and reagents solutions were dissolved at 40°C as recommended (“warm water”) and the ¹H-NMR spectrum was measured, the initial time for the reaction was counted from the moment that the tablet was completely dissolved. Then the samples ¹H-NMR were measured at close to the start and after 24 hours.

For the fixed temperature measurements, the tablet was dissolved at RT (22°C), 40°C and 60°C and the temperature maintained for the duration of the experiment and the measurements were taken every 20 minutes for the first 180 minutes and then at 360 minutes (6 hours) and a final measurement at 720 minutes (12 hours).

The pH measurements were performed, every three minutes for the first 15 minutes then at 20, 40, 60, 80 and 100 minutes using the pH meter and the solutions were made in H₂O instead of D₂O.

Subsequently, using all the data collected, a formulation was prepared following the tablet concentrations (see table below) for tetraacetylenediamine, sodium percarbonate and two different concentrations of citric acid to create two different pH environments one at the lower end of the optimal pH for optimal decomposition of peracetic acid at 5.5, CA200, and the other tablet pH 7-8 the CA100.

Table 5 – Main tablet components concentration in the tablet after dissolving in 150 ml, as recommended by the manufacturer and the stock solution prepared for each experiment.

| Ingredient | Tablet / Test concentration | | Stock solution (g/mL) |
|--------------------------------|-----------------------------|-----------------------|-----------------------|
| | g/ml | Mol/dm ³ | |
| TAED | 5.99×10^{-4} | 3.94×10^{-4} | 3.59×10^{-3} |
| NaPC | 1.44×10^{-3} | 1.38×10^{-3} | 8.64×10^{-3} |
| Citric Acid | 3.60×10^{-3} | 2.81×10^{-3} | 2.16×10^{-2} |
| Potassium Monopersulfate (MPS) | 2.16×10^{-3} | 2.13×10^{-3} | 1.30×10^{-2} |
| Sodium bicarbonate | 6.88×10^{-3} | 1.23×10^{-3} | 4.13×10^{-2} |
| Sodium carbonate | 1.73×10^{-3} | 2.45×10^{-3} | 1.04×10^{-2} |

The stock solutions concentrations shown in table 5, rightmost column, were prepared at 6x the original tablet concentration so upon mixture of all the components, in equal volumes, their final concentration will be equal to the total tablet and test formulation contents.

Table 6 – Tested solutions. Solutions prepared by mixing stock solutions at 6x test concentration. Polident and Placebo were dissolved as instructed by the manufacturer. (CA – citric acid, MPS - potassium monopersulfate)

| Stock | TAED | NaPC | CA | MPS | NaHCO ₃ | Na ₂ CO ₃ | H ₂ O |
|---------------|------|------|----|-----|--------------------|---------------------------------|------------------|
| Test Solution | | | | | | | |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 2 | 1 | 1 | 0 | 0 | 0 | 1 | 3 |
| 3 | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| 4 | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| 5 | 1 | 1 | 0 | 1 | 0 | 0 | 3 |
| 6 | 1 | 1 | 1 | 0 | 0 | 0 | 3 |
| Polident | 0 | 0 | 0 | 0 | 0 | 0 | 5 |

After mixing the ingredients following the table 6 specifications, the pH was measured, and then the best candidates with a pH between 5.5 – 8.2 were selected for subsequent analysis using H-NMR and the relevant changes against the tablet kinetics compared.

Table 7 – CA100 and CA200 solutions used to observe the pH effect on the TAED/NaPC system.

| Stock solution (mL) | TAED | NaPC | CA | H ₂ O |
|---------------------|------|------|--------|------------------|
| Test Solution | | | | |
| CA100 | 1 | 1 | 200 µL | 3.8 |
| CA200 | 1 | 1 | 400 µL | 3.6 |

In order to achieve the desired pH a small volume of the stock solution of citric acid shown in table 7 was added to a TAED / NaPC solution. The final concentration of citric acid in solution was for CA 100 7.2×10^{-4} g/mL corresponding to 9.37×10^{-5} mol/dm³ and for CA200 1.44×10^{-3} g/mL corresponding to 1.86×10^{-4} mol/dm³.

3.1.3 Results and Discussion

The results obtained were in conformity with the literature findings showing the relevance of the pH balance in the kinetics of the reaction between (tetraacetylenediamine) TAED and sodium percarbonate (NaPC) and in the stability of the resulting peracetic acid^{35,85}.

3.1.3.1 pH measurements studies

As stated in the previous section the solutions shown in table 6 and table 7 were prepared in duplicates of D₂O and H₂O. the pH measurements were performed using H₂O since it was cheaper and did not affect massively the reactions taking place.

The following table shows the pH values of the Polident tablet ingredients at product concentration.

Table 8 – Single ingredient solutions pH measurements (average of three measurements with the standard deviation).

| Ingredient | pH |
|----------------------------|--------------|
| citric acid | 1.65 ± 0.03 |
| polyethylene glycol | 6.80 ± 0.04 |
| potassium monopersulfate | 1.93 ± 0.03 |
| sodium benzoate | 7.01 ± 0.03 |
| sodium bicarbonate | 8.90 ± 0.04 |
| sodium carbonate | 11.67 ± 0.02 |
| sodium lauryl sulfoacetate | 7.37 ± 0.04 |
| TAED | 6.35 ± 0.02 |
| NaPC | 10.73 ± 0.04 |

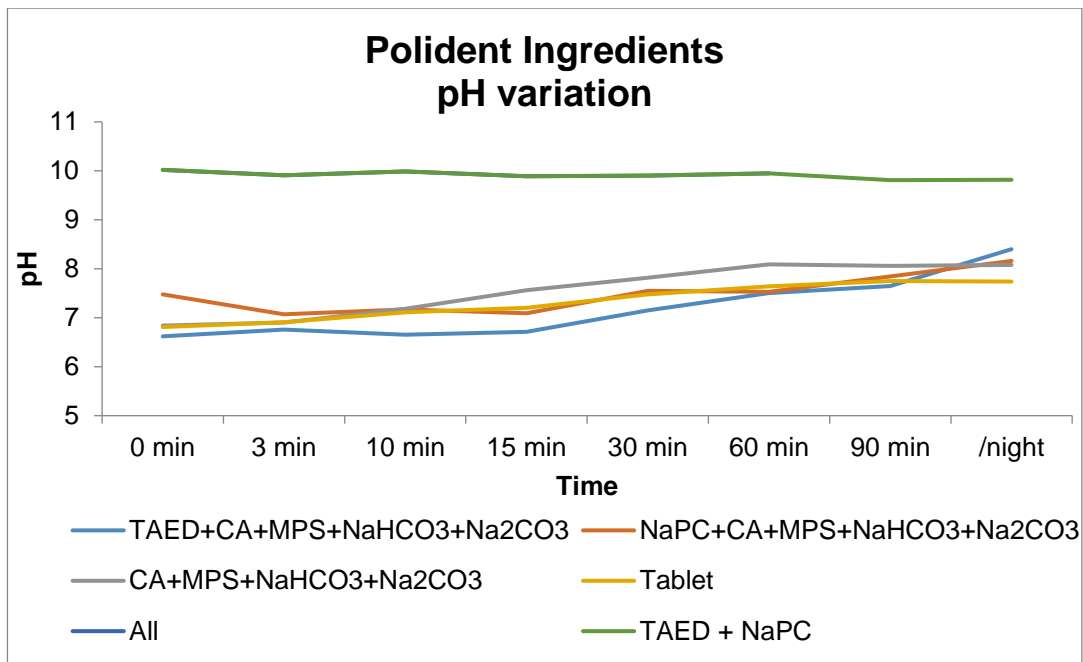


Figure 19 - Polident ingredients pH variation. Regular intervals pH measurements of solutions containing different combinations of Polident main ingredients TAED, NaPC, citric acid (CA), potassium monopersulfate (MPS), sodium carbonate (Na₂CO₃) and sodium bicarbonate (NaHCO₃).

Firstly, were observed the pH variation associated to different mixtures of the tablet ingredients.

The Figure 19 presents the pH of different solutions made using the main ingredients of the Polident tablet was measured. Test samples included

the main four ingredients (CA, Na₂CO₃, NaHCO₃ and MPS) in combination plus one or both main actives (TAED and NaPC). A tablet solution was also used for reference of the tablet conditions. The pH of the TAED/NaPC solution had a pH of 10, while all the other tested solutions had a pH around 6-8. As expected, when all the main ingredients and actives were mixed this showed the same pH as the tablet despite, the absence of some reactive ingredients like binders, fillers, colourants and flavours.

In a synergistic way the main ingredients plus actives contribute to the optimal pH found in the tablet. See table 8 for individual ingredients pH.

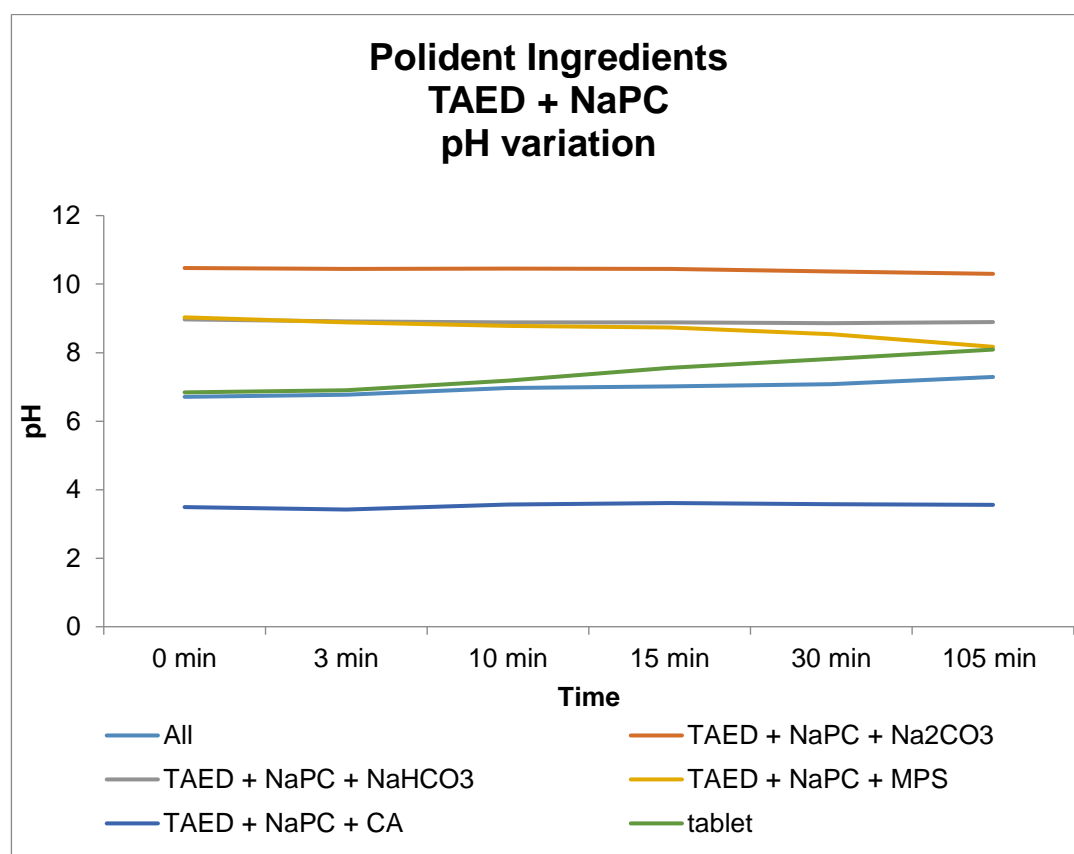


Figure 20 – pH variation of the TAED + NaPC solution plus single ingredients present in the Polident formulation vs Polident tablet, citric acid (CA), potassium monopersulfate (MPS), sodium carbonate (Na₂CO₃) and sodium bicarbonate (NaHCO₃)

The pH variation of solutions made with the main actives TAED and NaPC plus one of the main ingredients (CA, MPS, NaHCO₃, Na₂CO₃) was also measured at regular intervals for 105 minutes, Figure 20.

This result showed us that in the tablet formulation a combination of the ingredients at specific concentrations is necessary to achieve the optimal pH (7-8). Then on the last pH experiment we simplified the formulation to only three ingredients, the main actives TAED, NaPC and citric acid to lower the pH of the TAED/NaPC solution, close to the optimal levels 5.5 – 8.2.

During the Polident formulation pH studies it was observed that, at the tablet concentration a mixture of all the main ingredients resulted in a solution with a pH around 6.9 despite the fact that the single solutions at the tablet concentration showing different pH (see Table 8). Since the TAED and NaPC combined solution presented a high pH around 10 and citric acid solution had a pH of 1.65, it was decided to use citric acid to regulate the pH of the main actives and observe the effects of the pH in the reaction between TAED and NaPC and decomposition of its products.

Two new formulations were developed, it is important to keep present that the reaction between TAED and H_2O_2 can occur at a pH much higher than the optimal level (5.5 – 8.2) but the final products do not lead to the spontaneous decomposition of peracetic acid thus not generating ROS and also that the decomposition of H_2O_2 is enhanced by a higher pH and temperature.

Three solutions were used CA100, CA200 and a solution of TAED and NaPC see table 8 with the results of the pH measurements shown in the following Figure.

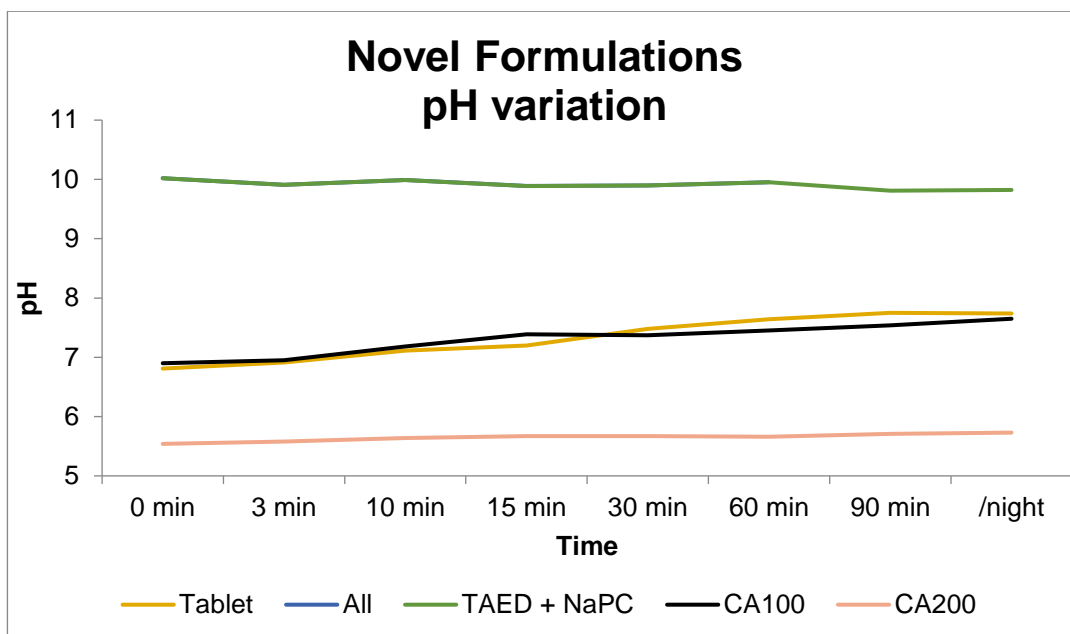


Figure 21 – pH variation of the TAED + NaPC, tablet, CA100, CA200 and a combination of the six main ingredients (TAED, NaPC, CA, MPS, Na_2CO_3 , NaHCO_3).

As observed in the previous results (Figures 19 - 21) the pH of each of the solutions did not vary drastically throughout the experiments. The CA200 solution had a pH of 5.5, the lower end of the optimal pH for the spontaneous decomposition of peracetic acid, CA100 a pH between 7-8, the top end of the optimal range. While the TAED and NaPC solution pH was outside of the optimal pH range at 10. The combination of the 6 main ingredients had a similar pH as the tablet and CA100.

After determining that the pH observed were on the ranges that we wanted to study, within the optimal pH for the spontaneous decomposition of peracetic acid 5 - 8, the studies moved to the next phase and the samples were studied using quantitative $^1\text{H-NMR}$ as described in the methods section.

3.1.3.2 Proton NMR studies

First a solution of TAED/NaPC alongside single ingredient solutions were analysed to pinpoint where to expect the NMR peaks of main products namely Tri-AED, DAED, peracetic acid and the main ingredients expected in the tablet solution $^1\text{H-NMR}$. Since the TAED/NaPC pH during the reaction was around 10 and such pH favours a rapid H_2O_2 decomposition to HOO^- and H^+ leading to a rapid perhydrolysis of TAED therefore the TAED peaks (CH_3 - 2.43 ppm, CH_2 - 3.94 ppm, Figure 27, were not detected in this experiment. The peak at 4.8 ppm corresponds to the deuterium oxide used as a solvent (D_2O) and it is present throughout all the spectra.

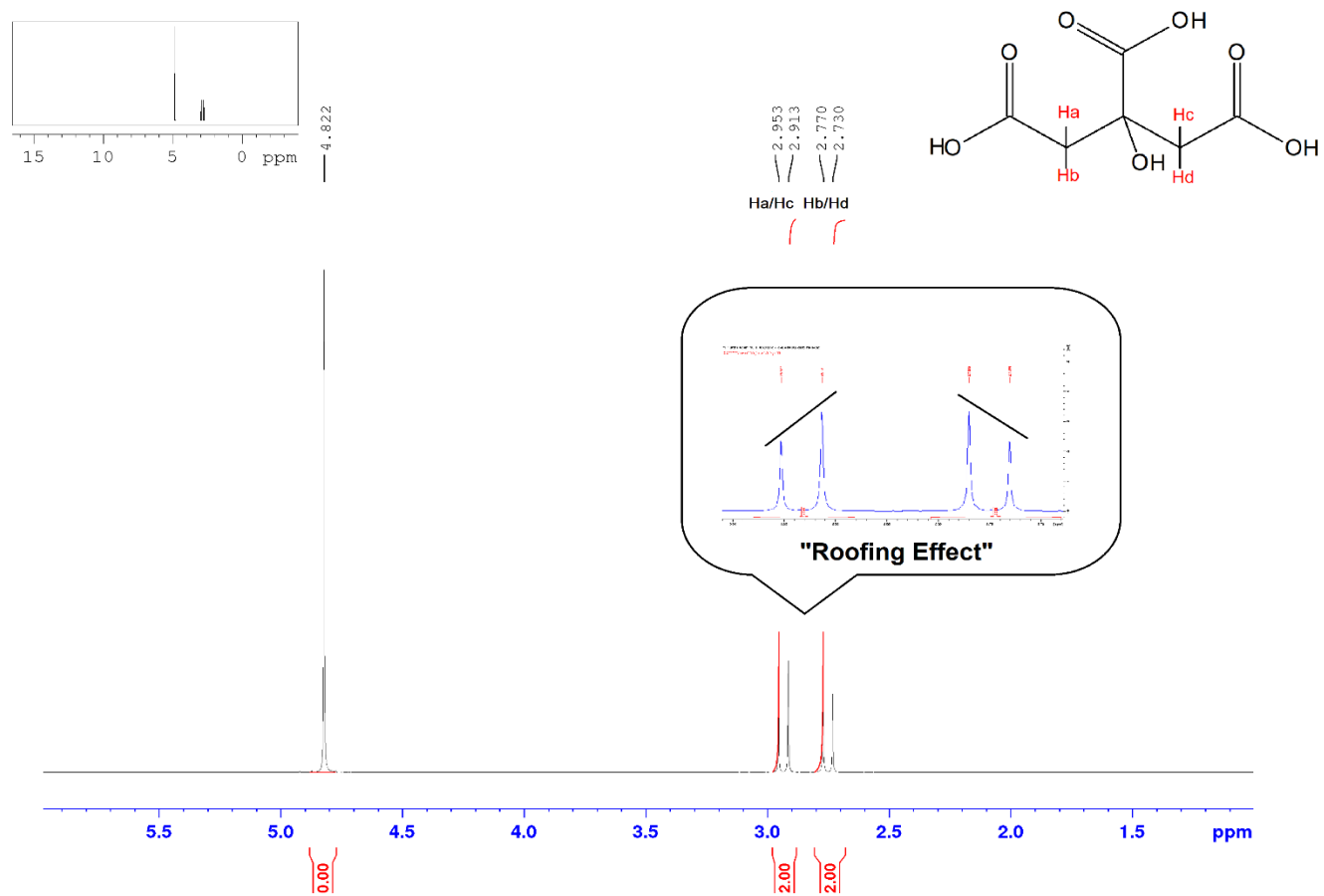


Figure 22 – Citric acid ^1H -NMR spectra. Two doublets sloped (roofing) with a coupling constant around 15.89 Hz (four peaks) 2.95, 2.91, 2.77, 2.73 ppm, see figure for assignment. The peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D_2O .

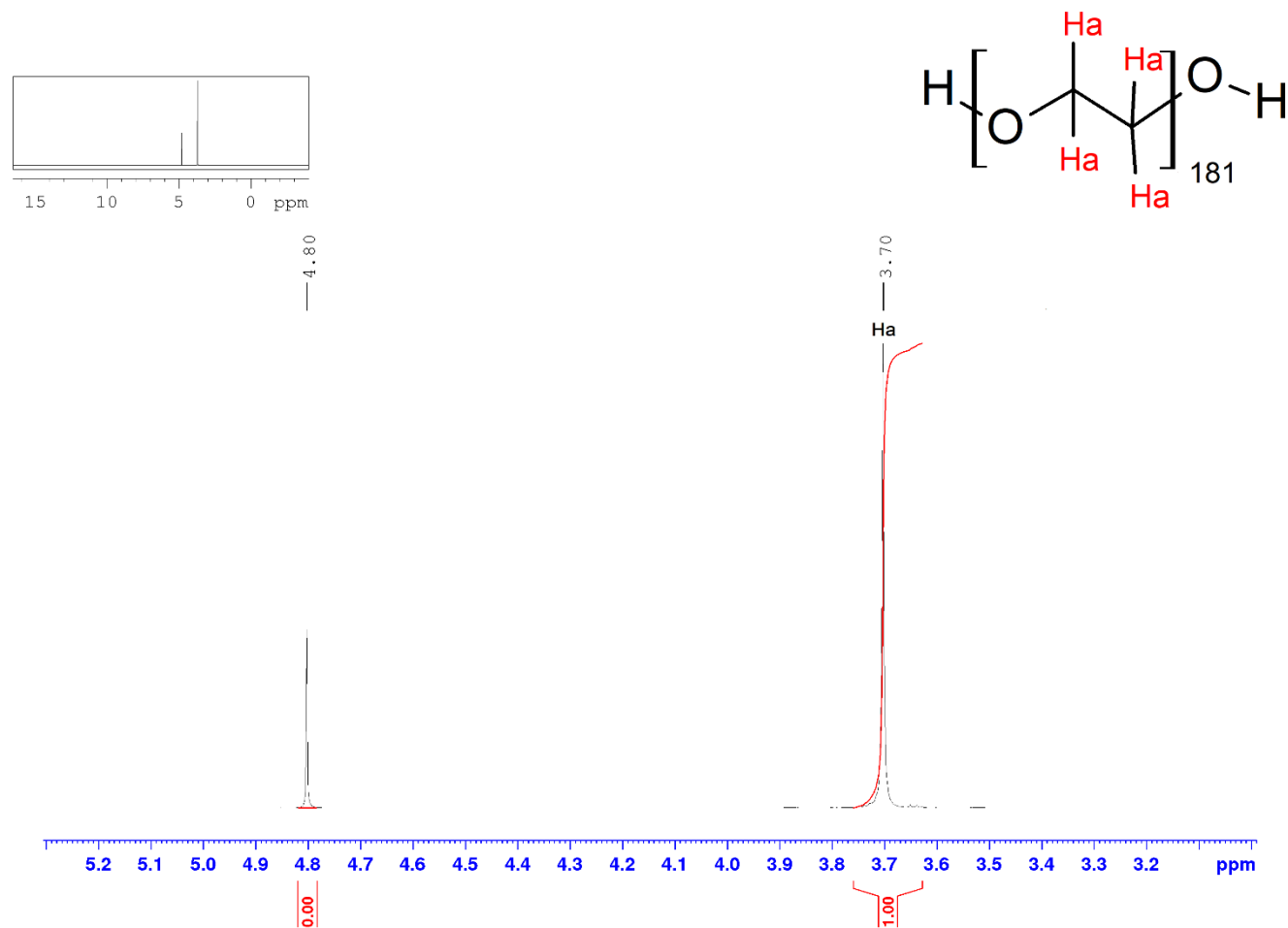


Figure 23 – Polyethylene glycol $^1\text{H-NMR}$ spectra. The peak at 3.70 ppm corresponds to the CH₂ groups of the polymer (724 protons). The peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D₂O.

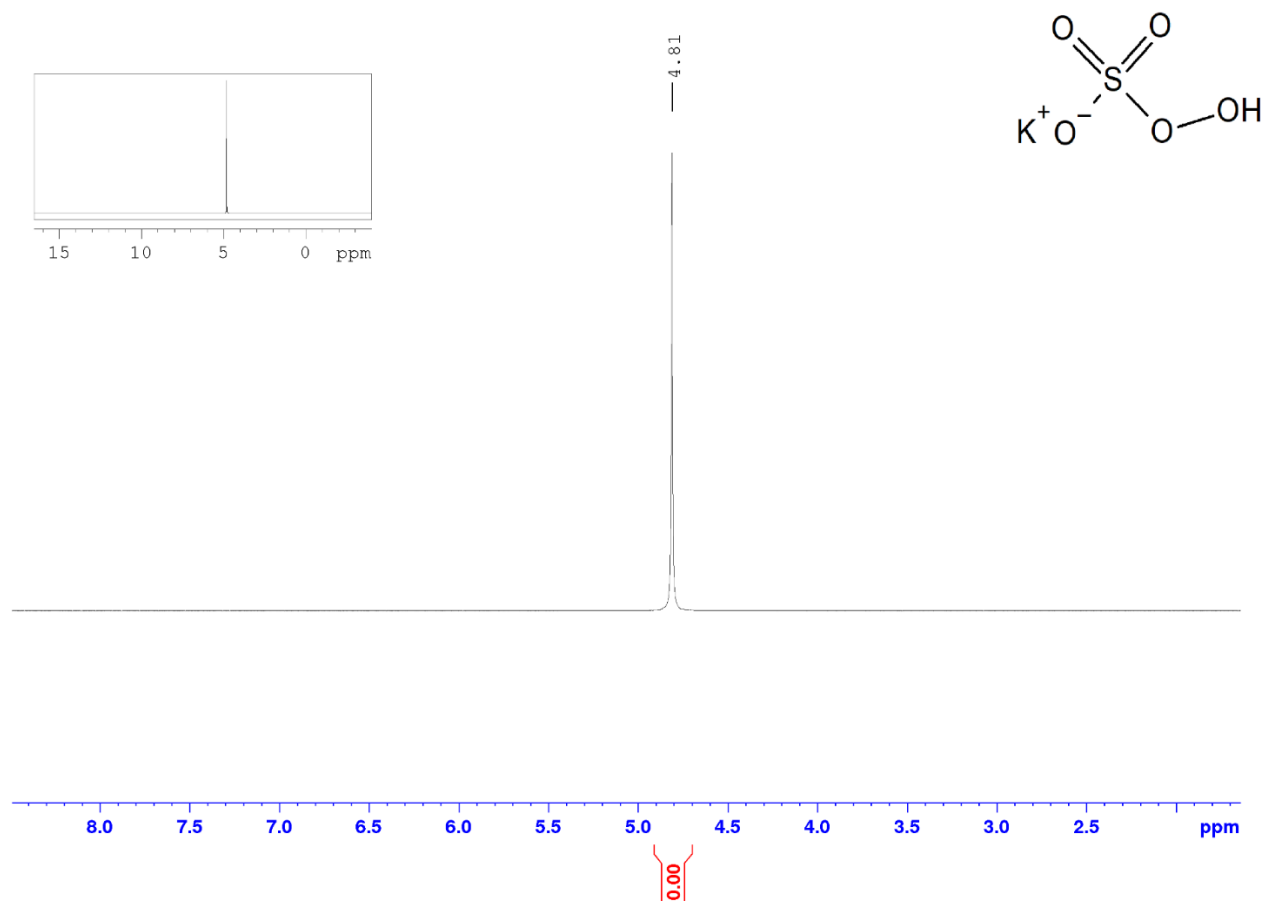


Figure 24 – Potassium monopersulphate $^1\text{H-NMR}$ spectra. No protons were detected because of exchange of the acidic protons with deuterium. The peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D_2O .

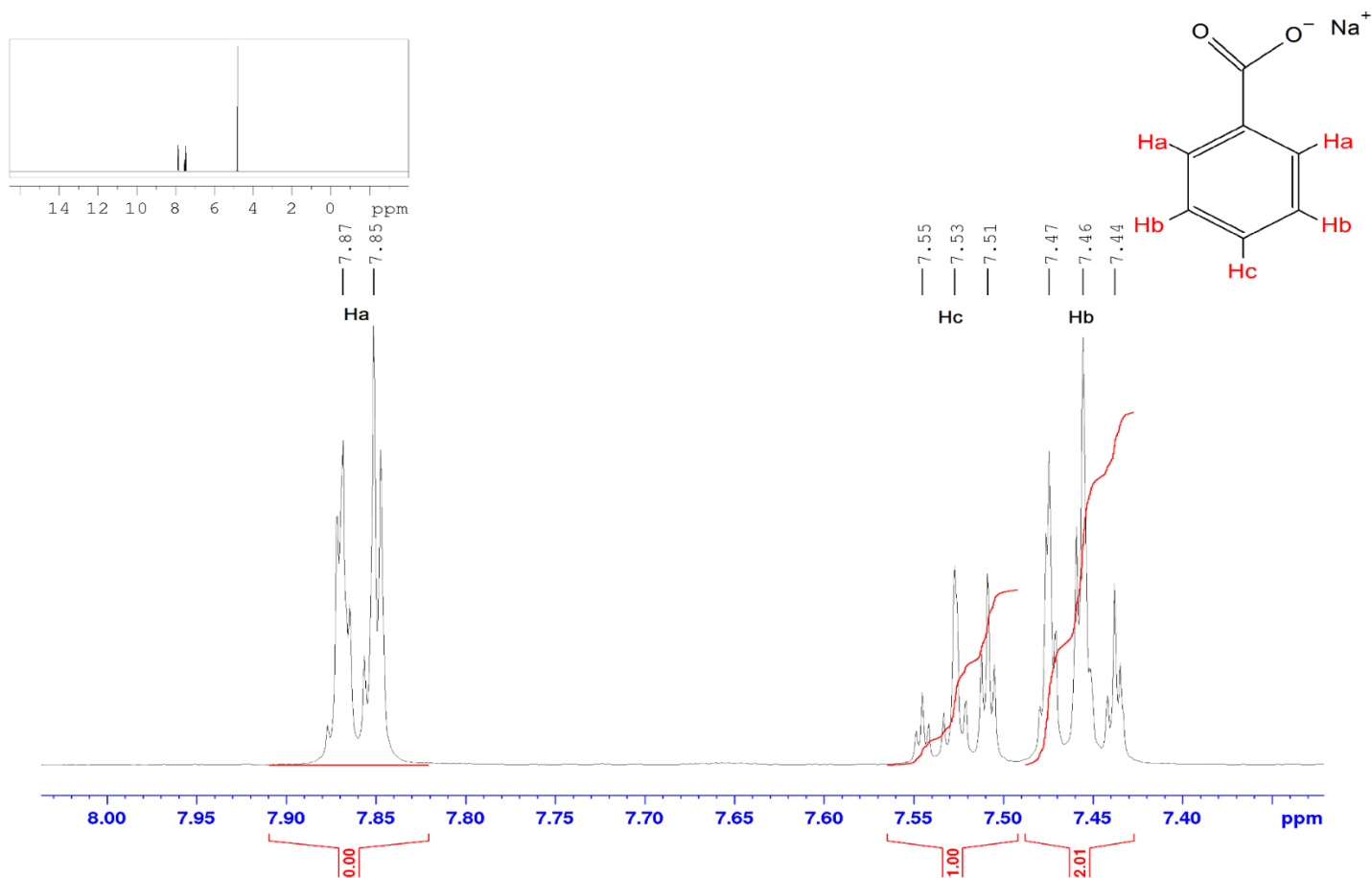


Figure 25 – Sodium benzoate ¹H-NMR spectra. Three regions were identified, the Ha protons (see figure) originated a doublet at 7.85 – 7.87 ppm, while Hc and Hb produced one triplet of triplets (multiplets) each at 7.50 – 7.55 ppm and 7.44 – 7.47 ppm respectively. The integration value of 0.00 corresponds to the calibrate value of 2.

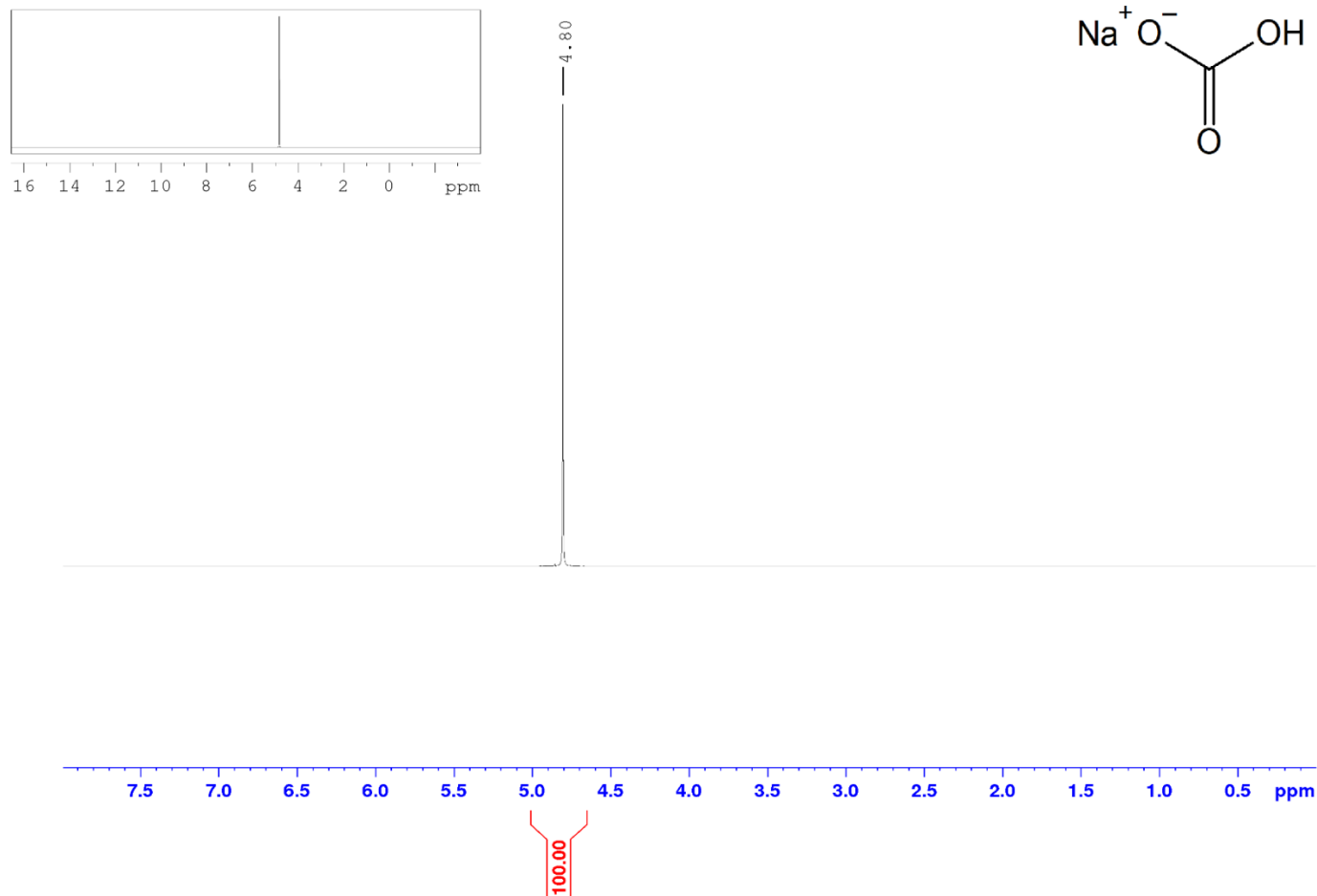


Figure 26 – Sodium bicarbonate $^1\text{H-NMR}$ spectra. No protons were detected because of exchange of the acidic protons with deuterium, the peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D_2O .

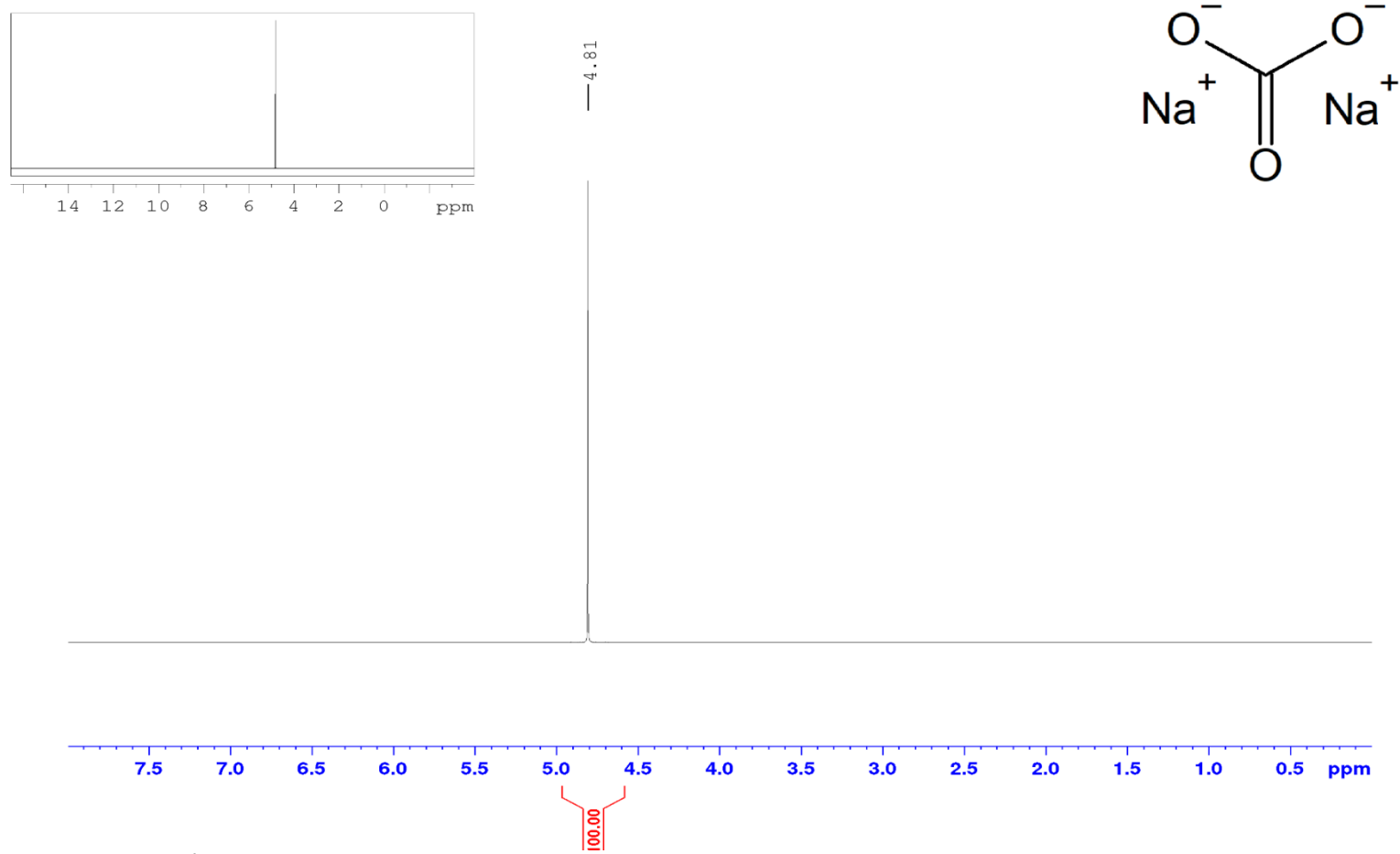


Figure 27 - Sodium carbonate $^1\text{H-NMR}$ spectra. No protons were detected because of exchange of the acidic protons with deuterium, the peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D_2O .

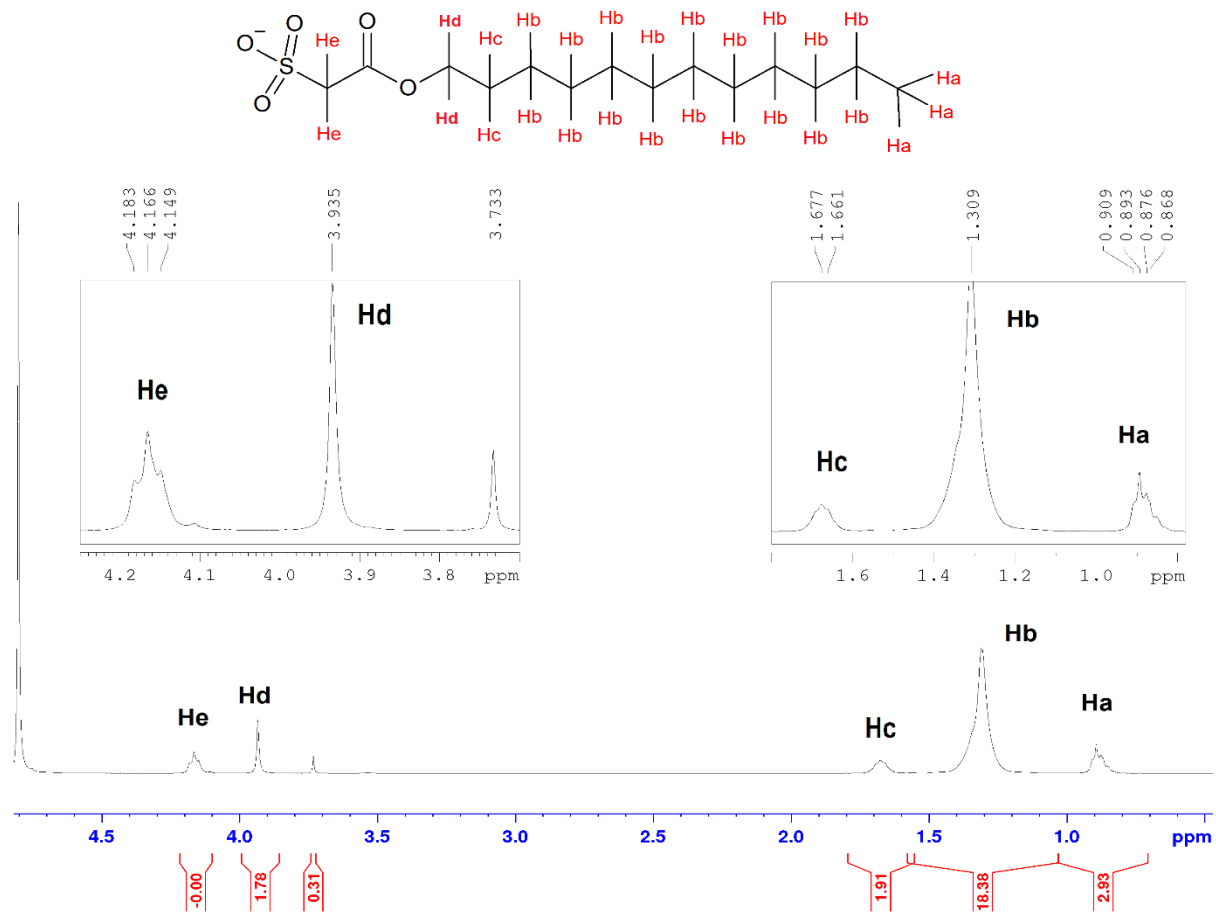


Figure 28 - SLSOA ¹H-NMR spectra. Six regions identified, see figure for peak assignment. The peaks identified as Hb should appear as several multiplets but due to the 400MHz used and only 8 scans resulted in a spectrum with poor resolution. The integration value of 0.00 corresponds to the calibrate value of 2.

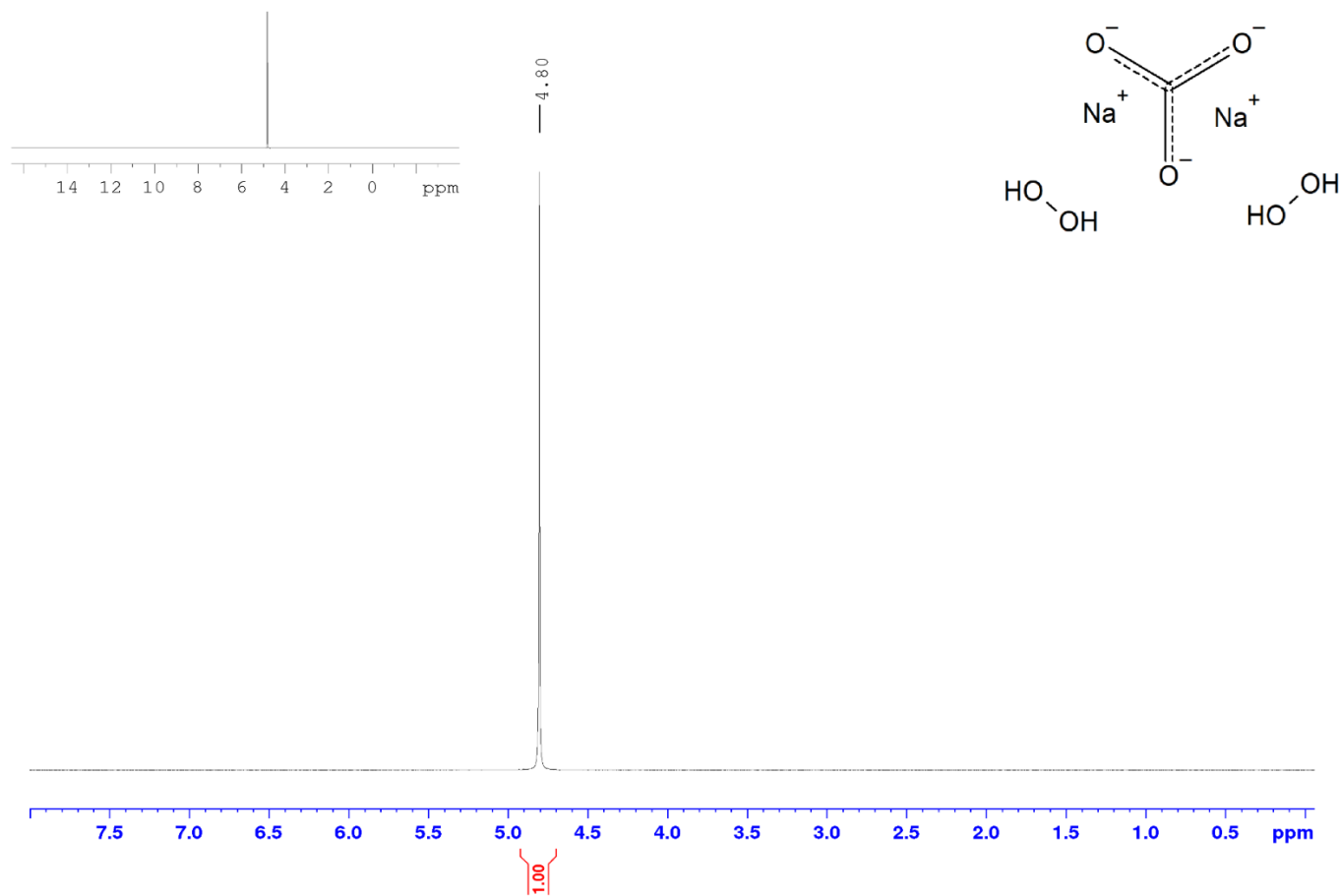


Figure 29 - Sodium percarbonate $^1\text{H-NMR}$ spectra. No protons were detected because of exchange of the acidic protons with deuterium. The peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D_2O .

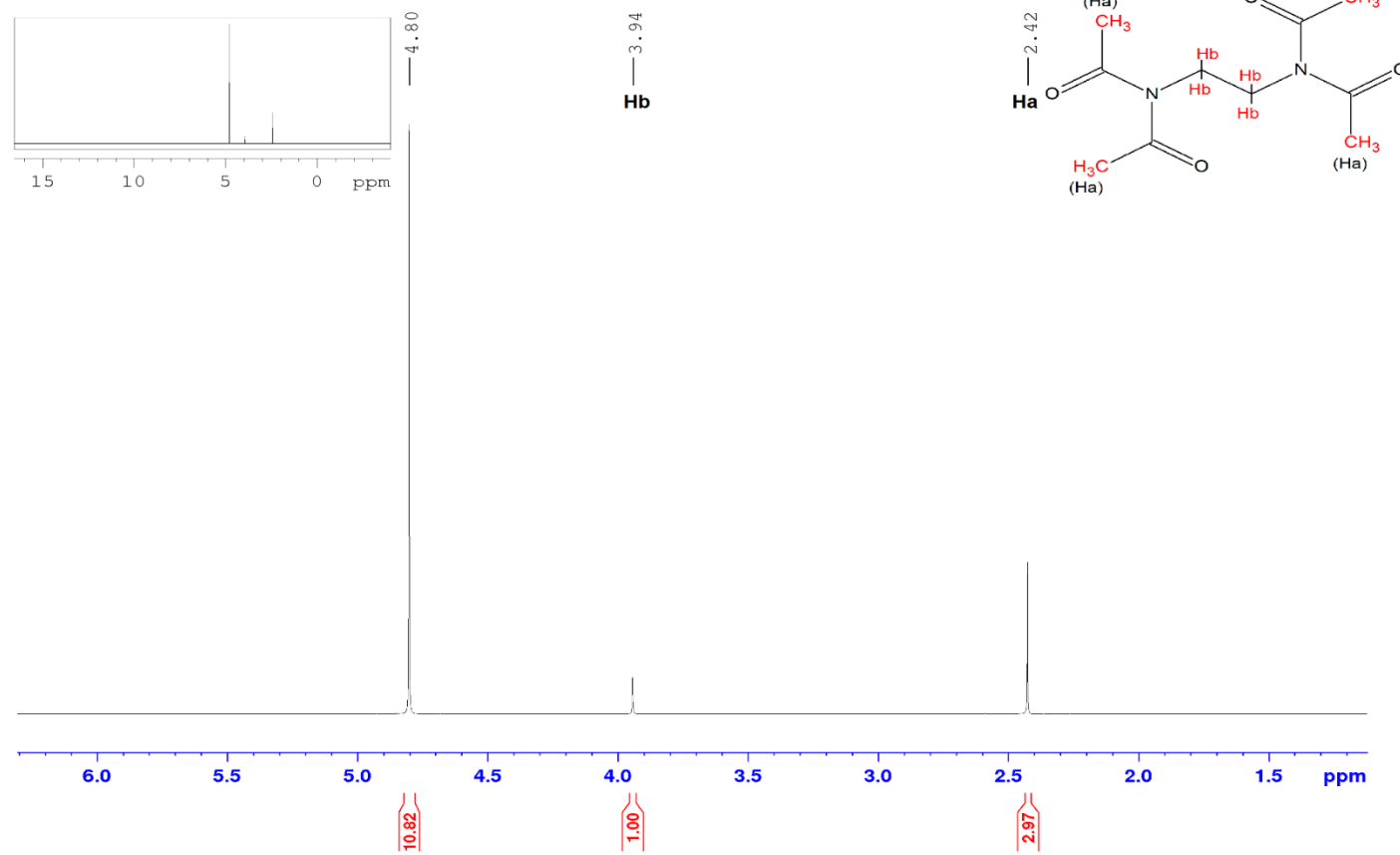


Figure 30 - TAED ¹H-NMR spectra. Two regions identified see figure for peak assignment, 3.94 ppm (s) CH₂ groups (4H) and 2.42 ppm (s) CH₃ groups (12H) of TAED. The peak at 4.80 ppm corresponds to deuterium oxide used as solvent resulting from the residual hydrogen impurities present in D₂O.

Table 9 - NMR peaks assigned to the ingredients and products (s – singlet, d – doublet, t – triplet, m – multiplets)

| | Compound | Protons detected | Type | Peaks / Group (ppm) | Figure |
|--------------------|--------------------------|------------------|-----------------------|--|-----------------------------|
| Ingredients | Deuterium oxide | | s | 4.81 | Solvent peak in all figures |
| | Citric Acid | 4 | d d | 2.95, 2.91 CH ₂ 2.77, 2.73 CH ₂ | 22 |
| | Polyethylene Glycol | 724 | s | 3.70 CH ₂ | 23 |
| | Potassium Monopersulfate | 0 | -- | -- | 24 |
| | Sodium Benzoate | 5 | m m m | 7.85 – 7.87 7.50 – 7.55 7.44 – 7.47 | 25 |
| | Sodium Bicarbonate | 0 | -- | -- | 26 |
| | Sodium Carbonate | 0 | -- | -- | 27 |
| | SLSOA | 27 | m m t s t | [0.87-0.91] 1.31 1.68 3.94 4.17 | 28 |
| | Sodium Percarbonate | 0 | -- | -- | 29 |
| | TAED | 16 | s s | 3.94 CH ₂ 2.42 CH ₃ | 30,36 |
| Products | Tri-AED | 13 | t t s s | 3.86 CH ₂ 3.40 CH ₂ 2.41 CH ₃ 1.95 CH ₃ | 34,36 |
| | DAED | 10 | s s | 3.28 CH ₂ 1.96 CH ₃ | 34,36 |
| | Peracetic Acid | 3 | s | 2.1 CH ₃ | 31,36 |
| | Acetic Acid | 3 | s | 1.91 CH ₃ | 31,36 |

The spectra shown in Figures 22 - 30 were valuable to help identify each of the ingredients peaks and predict their chemical shift in order to assign the peaks resulting from the tablet solution. The Table 9 presents the $^1\text{H-NMR}$ peaks observed throughout the experiments and used in the determination of the concentrations of each of the compounds detected.

Citric acid, polyethylene glycol, sodium benzoate, SLSOA, and TAED had a $^1\text{H-NMR}$ spectra while sodium bicarbonate, sodium carbonate and sodium percarbonate did not display $^1\text{H-NMR}$ spectra due to the absence of detectable protons because of exchange of the acidic protons with deuterium therefore due to the different spin of deuterium the protons are not seen.

One interesting feature to note was the “roofing” observed in the citric acid spectra Figure 22, when two doublets slope one towards the other, this happens due to the fact that the protons in the methylene group are diastereotopic, identical groups attached to a chiral centre¹¹⁷ the coupling constant within the doublets show that both peaks are related and belong to groups related to each other. Other interesting spectra was the SLSOA, shown in Figure 28, where for some protons the 400 MHz and 8 scans were insufficient to resolve all the protons environments. The region from 1.21 ppm to 1.45 ppm contains broad peaks that would be resolved using a more powerful NMR and by increasing the number of scans^{114,118}.

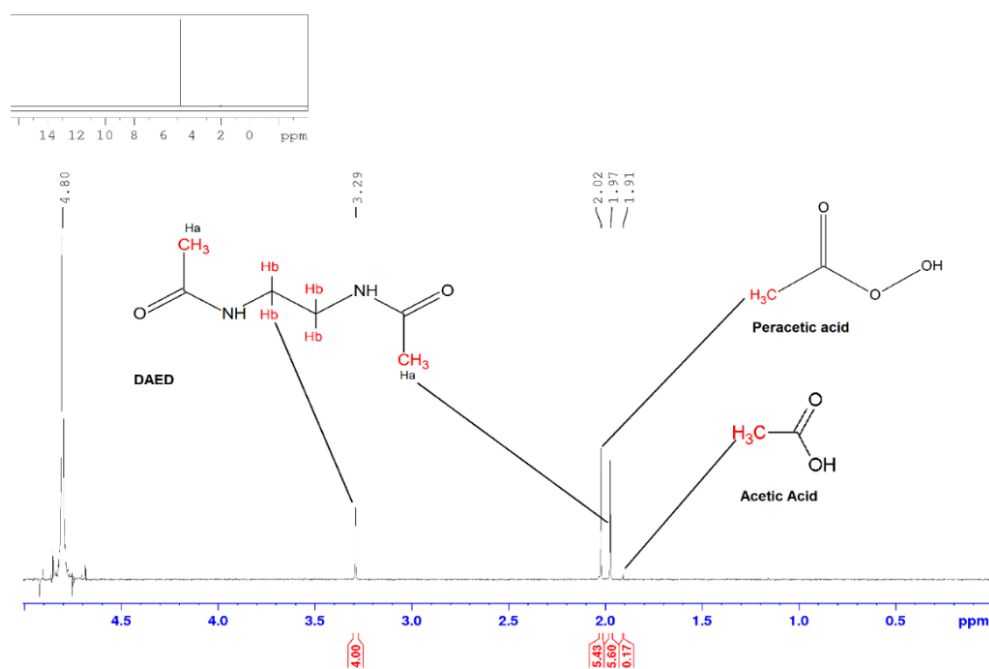


Figure 31 – TAED and NaPC solution $^1\text{H-NMR}$ spectra, see figure for peak assignment. The peaks of TAED 3.94 (s), 2.42 (s) shown on Figure 30 were not detected in this experiment.

Following the determination of the $^1\text{H-NMR}$ chemical shifts of the protons present in each of the ingredients tested we progressed to perform the same study using a solution containing TAED and NaPC to determine the chemical shift of the products of our main reaction, Figure 31.

The following graphs present the kinetic studies using the TAED and NaPC solution. It will be evident that the DAED peaks do not change its intensity (integration) between timepoints. The only observed changes are between peracetic acid (PAA) and acetic acid (AA) peaks. In the final timepoint (overnight) most of the PAA was converted into AA.

These results were essential to interpret and analyse the Polident tablet solution $^1\text{H-NMR}$ spectra and reactions taking place. It showed the relative chemical shift of the products of the perhydrolysis of TAED namely DAED, AA, PAA and the conversion between peracetic acid into acetic acid was also evident.

The spectrum shown in Figure 32 top, identified as the initial time point but, due to instrument limitations the measurement only occurred 5

minutes after mixing the ingredients. The peaks were assigned corresponding to DAED, CH₂ groups at 3.28 ppm and 1.96 ppm for the two CH₃ groups, to the peracetic acid CH₃ group 2.01 ppm and final product acetic acid CH₃ groups at 1.90 ppm.

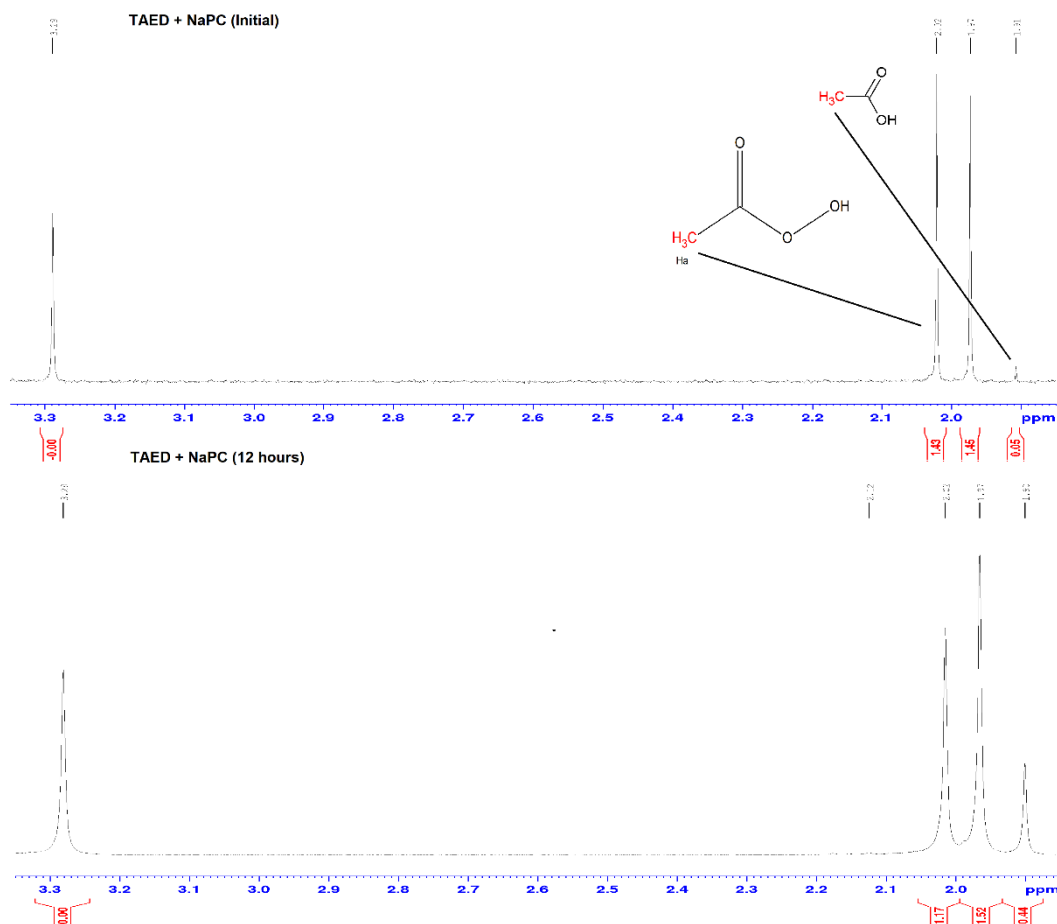


Figure 32 - TAED + NaPC solution spectra initial and 12 hour time points evidencing the conversion of peracetic acid into acetic acid.

As aforementioned due to the high pH that promotes the rapid decomposition of H₂O₂ and subsequent perhydrolysis of TAED it was not possible to observe and follow the decomposition of TAED into Tri-AED and DAED. But the experiment allowed us to see the conversion of peracetic acid into acetic acid. This was observed by the changes in the corresponding peaks intensity shown by the integration of the area covered by the peak. On the following figures, the ¹H-NMR spectra for the Polident tablet solution is shown. The following Figure 33 shows the overall spectra with all the

detectable ingredients, reagents and products, for an easy read only the ingredients are shown in the first spectra. The Figure 34 shows the region from 2 to 5 ppm where TAED and the reaction products are localized. From this point forward we moved on to determine the area of each peak (peak integration) to quantify the protons giving rise to the signal and determine the reagent or product concentration.

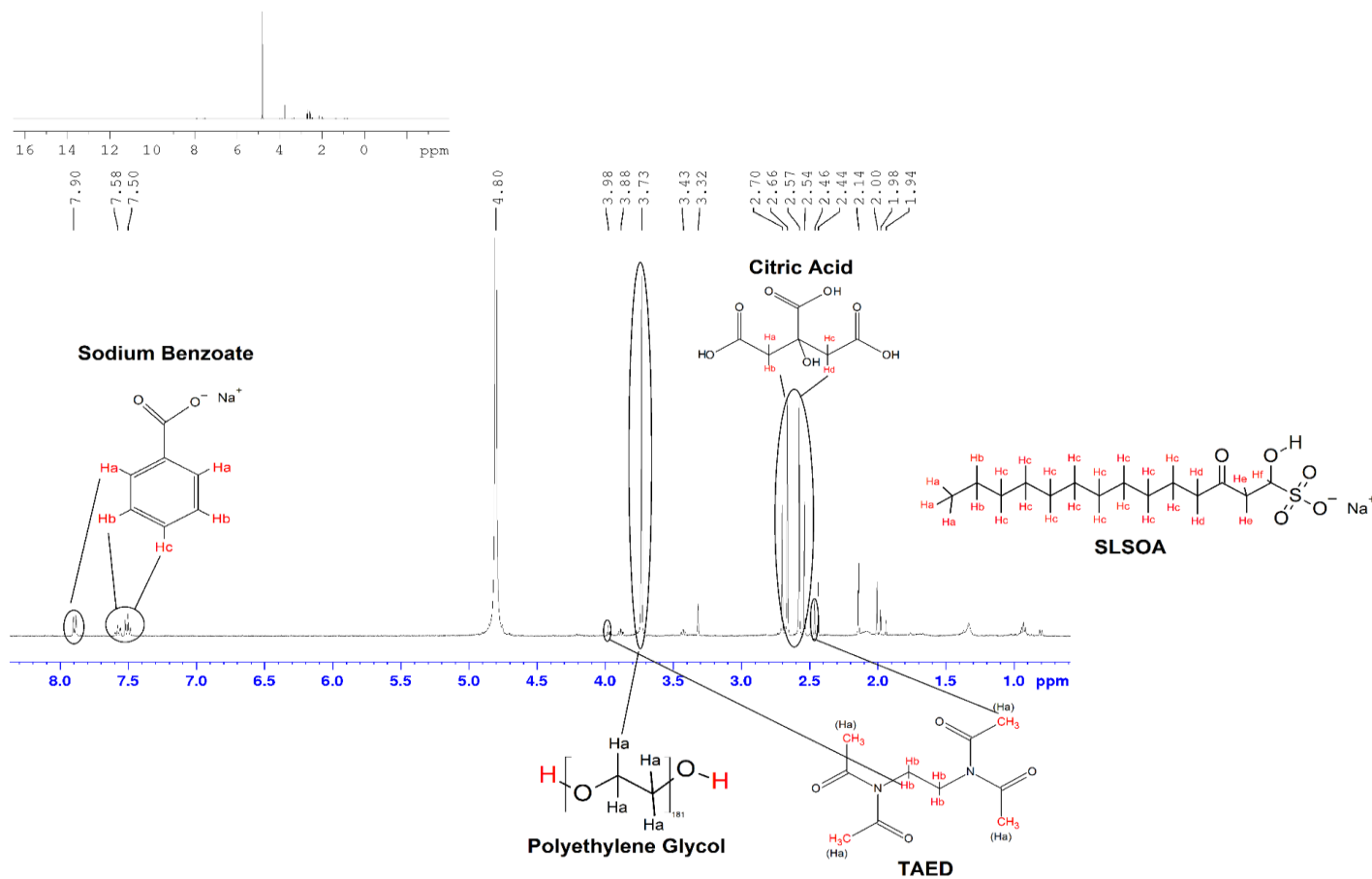


Figure 33 – Polident tablet solution ¹H-NMR spectra identifying the starting materials. The SLSOA peaks were not identified to keep the schematic easy to read. The non-identified peaks in the region from 2-4 pm correspond to the products and TAED shown in more detail in the Figure 34.

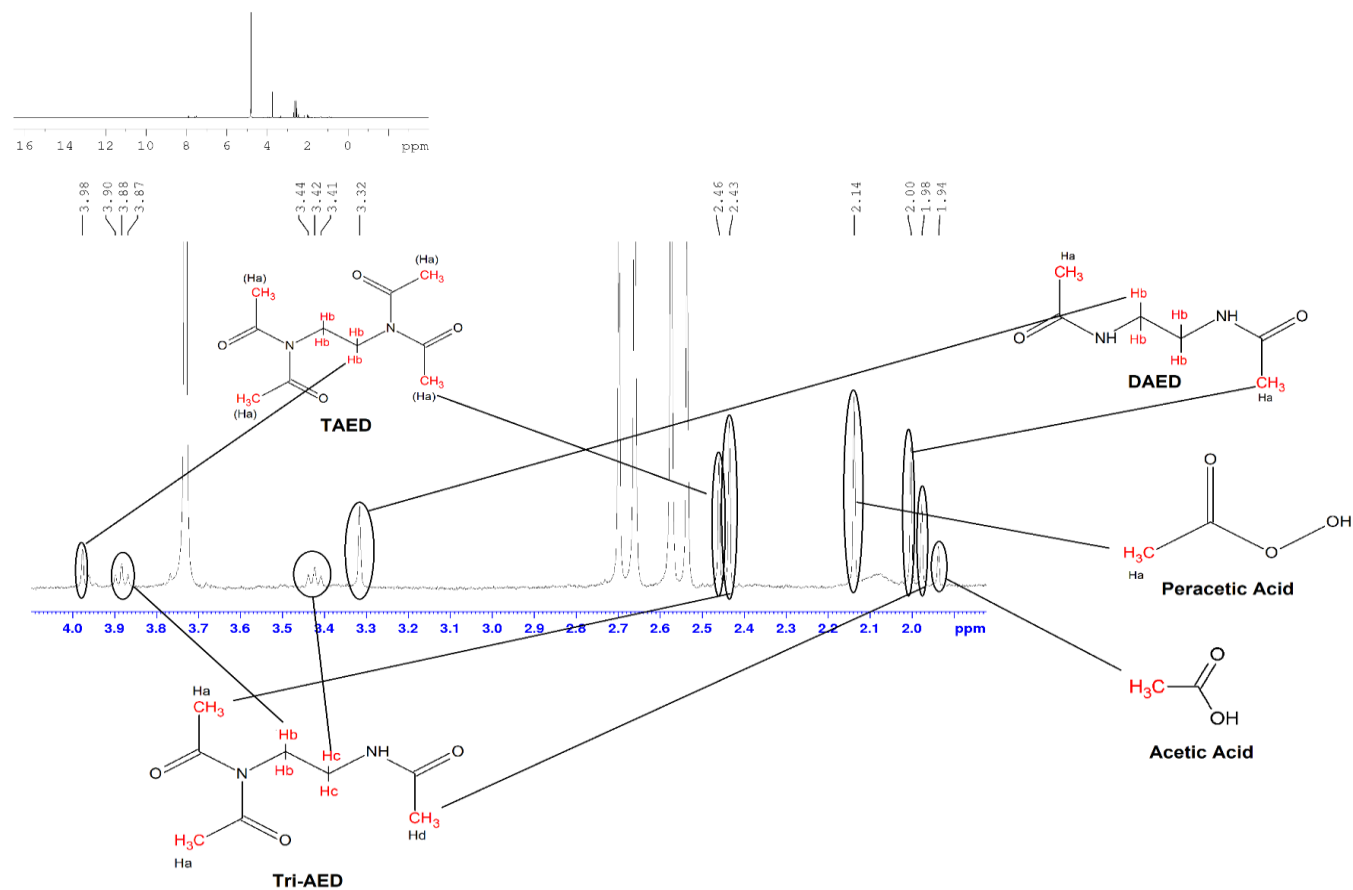


Figure 34 – Polident tablet solution $^1\text{H-NMR}$ spectra with peak assignment of the region from 2 – 4 ppm showing TAED and the reaction products. The non-identified peaks at 2.5 – 2.75 ppm and 3.7 ppm correspond to citric acid and polyethylene glycol respectively.

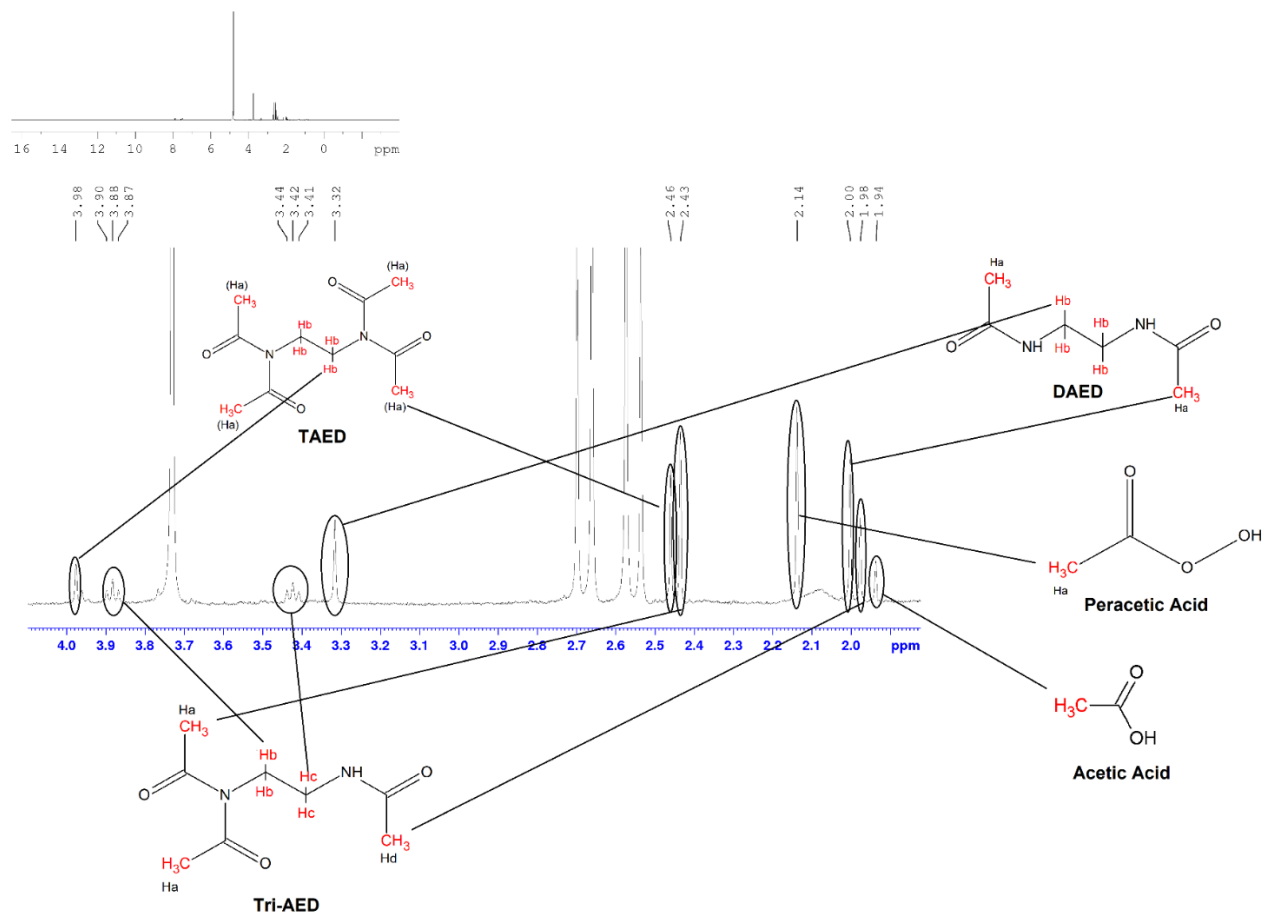


Figure 35 – Polident tablet solution ¹H-NMR spectra with peak assignment of the region from 2 – 4 ppm showing TAED and the reaction products. The non-identified peaks at 2.5 – 2.75 ppm and 3.7 ppm correspond to citric acid and polyethylene glycol respectively.

The peaks that were detected and assigned in the Figures 34 and 35 were citric acid two sloped doublets 2.47 ppm – 2.69 ppm corresponding to 2 protons each, polyethylene glycol one high intensity peak at 3.69 ppm corresponding to 724 protons; sodium benzoate two triplets in the region between 7.44 – 7.54 ppm (3 protons) and a doublet at 7.84 ppm – 7.86 ppm (2 protons); acetic acid one peak corresponding to 3 protons, at 1.71 ppm, peracetic acid one peak at 2.09 ppm (3 protons), TAED two peaks CH₃ groups 2.41 ppm (6 protons), CH₂ groups 3.93 (4 protons), Tri-AED two peaks corresponding to the CH₃ groups at 1,90 ppm (3 protons) and 2.39 ppm (6 protons) and two triplets one for each of the CH₂ groups at 3.37 to 3.40 ppm and at 3.82 to 3.85 ppm with 2 protons each; DAED presented two peaks one from the CH₃ group at 1.96 ppm originating from 6 protons and CH₂ groups at 3.27 ppm belonging to 4 protons.

In order to correctly assign the peaks to the protons originating it the area under the peaks was first calculated by integrating the peak using the software Bruker Topspin 4.0.5 and compared within the compounds to ensure that the protons being analysed belonged to the compounds being studied. By checking the ratios between the types of protons detected within each molecule. This same principle is the root of qNMR (quantitative NMR) used to determine the concentration of components within a mixture. The intensity of the NMR signal is proportional to the number of nuclei giving origin to it. Therefore, if we determine the area under a curve and know the concentration of one of the components within the mixture we can effectively determine the concentration of the other components in solution¹¹⁸. Not all the ingredients present, in the tablet, were detected by ¹H-NMR mainly due to the low concentration of some compounds in the tablet. The OH groups appeared to have been deuterated by the D₂O used as solvent so were not observed. This result is a minor issue since it did not affect our capabilities to detect the relevant species present in solution and it was the closest solvent that would mimic the natural conditions of how the consumer would use the product. The results obtained can be improved by increasing the number of scans that are performed on the sample and by using a more

powerful NMR equipment that operates at higher frequency thus increasing the resolution of the $^1\text{H-NMR}$ “picture”.

3.1.3.3 Kinetic Studies of Polident

Following the pH and NMR studies. The following studies involved once more $^1\text{H-NMR}$ but in this instance the solutions were continuously measure for fixed periods of time.

The Figure 36 shows the $^1\text{H-NMR}$ spectra of real time with the initial time point (5 minutes after tablet dissolution), 6 hours and 12 hours. In this experiment the tablet was dissolved in D_2O at 40°C and the measurements were taken at room temperature (approx. 23°C) to mimic how the consumer uses the product. There were observed and tracked the two step perhydrolysis of TAED, with the presence of the corresponding intermediary tri-AED with peaks at 3.86 ppm (t), 3.40 ppm (t) both corresponding to both CH_2 groups, 2.41 ppm (s) corresponding to two CH_3 groups and 1.95 ppm (s) corresponding to a CH_3 group. A decrease in the intensity of the peaks corresponding to both compounds was observed alongside with an increase in the DAED peaks intensity at 3.28 ppm and 1.98 ppm corresponding to the CH_2 groups and CH_3 groups respectively. It was also evident the conversion between peracetic acid at 2.11 ppm and acetic acid 1.91 ppm that occurred at a slower rate than in the solution between TAED and NaPC used alone.

This experiment showed that the only peaks that significantly changed in intensity corresponded to TAED related compounds that increased and decreased according to the reaction progress and peracetic acid and acetic acid. Tri-AED started to appear, and its concentration increased and decreased once DAED was being produced. DAED concentration increased throughout the duration of the experiment until reaching a plateau corresponding to the initial concentration of TAED, peracetic acid and acetic acid peaks also varied throughout the experiment. The remaining peaks detected, polyethylene glycol, citric acid and sodium

benzoate peaks intensities remained “constant”, with minor variations due to the inaccuracies intrinsic to the instrument measurements.

The plot of the \ln of the concentration of TAED vs time (Figure 38) shows that the TAED decomposition follows a first order kinetic reaction meaning that the decomposition of TAED under these circumstances, with excess of H_2O_2 , only depends on its concentration with a rate constant of 0.0167 s^{-1} .

Upon dissolution of the tablet the formation rate of acetic acid was 3 uM per minute shown by the equation in Figure 37. In an ideal case scenario, where spontaneous decomposition is the only decomposition route, each two moles of acetic acid generated gives rise to one mol of singlet oxygen.

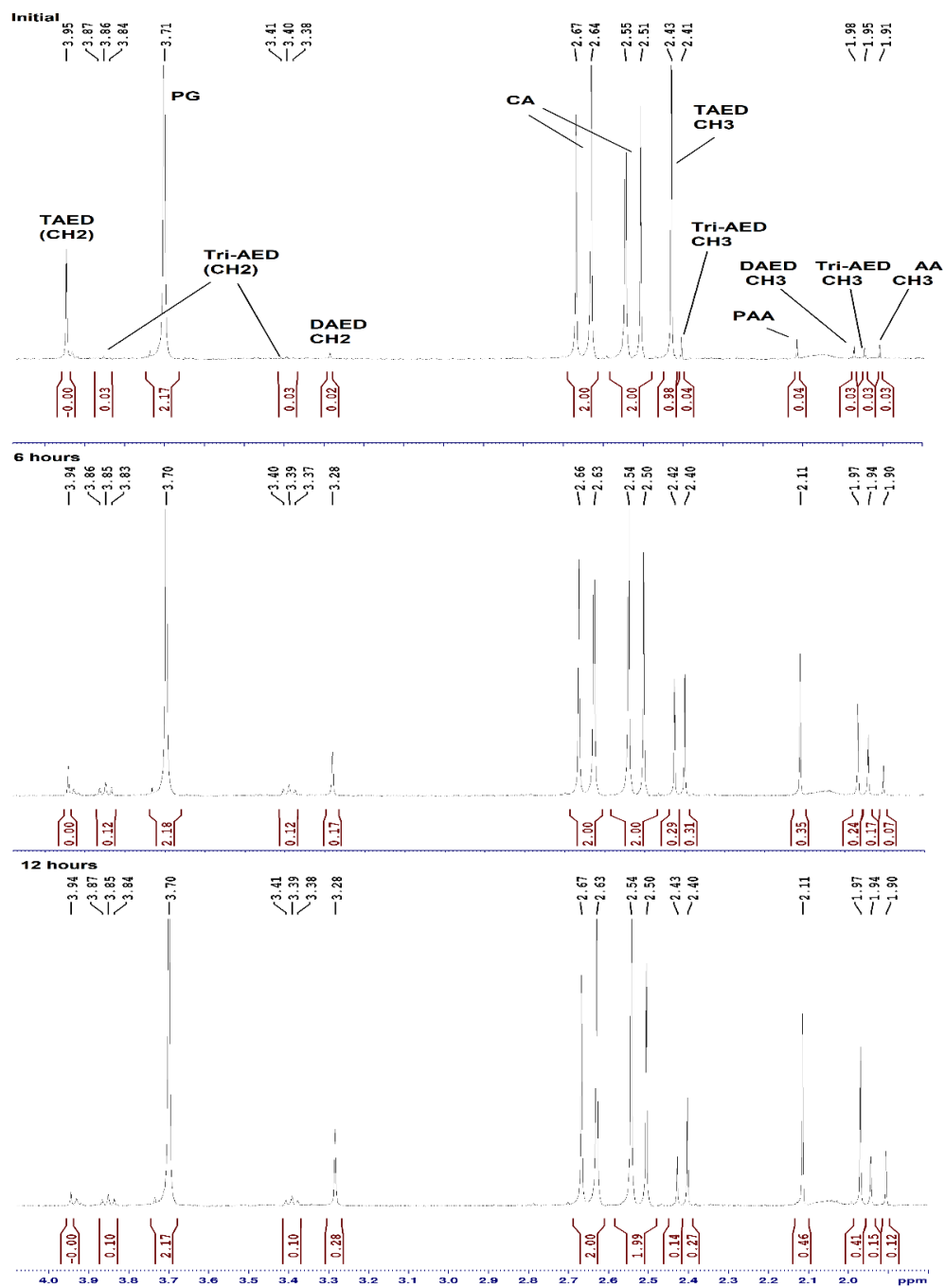


Figure 36 – Polident tablet solution ¹H-NMR spectra of kinetic measurements, top initial, middle 6 hours, bottom 12 hours. The black number on top of each spectra correspond to the chemical shift in ppm and the red at the bottom the peak integration calibrated using citric acid as reference.

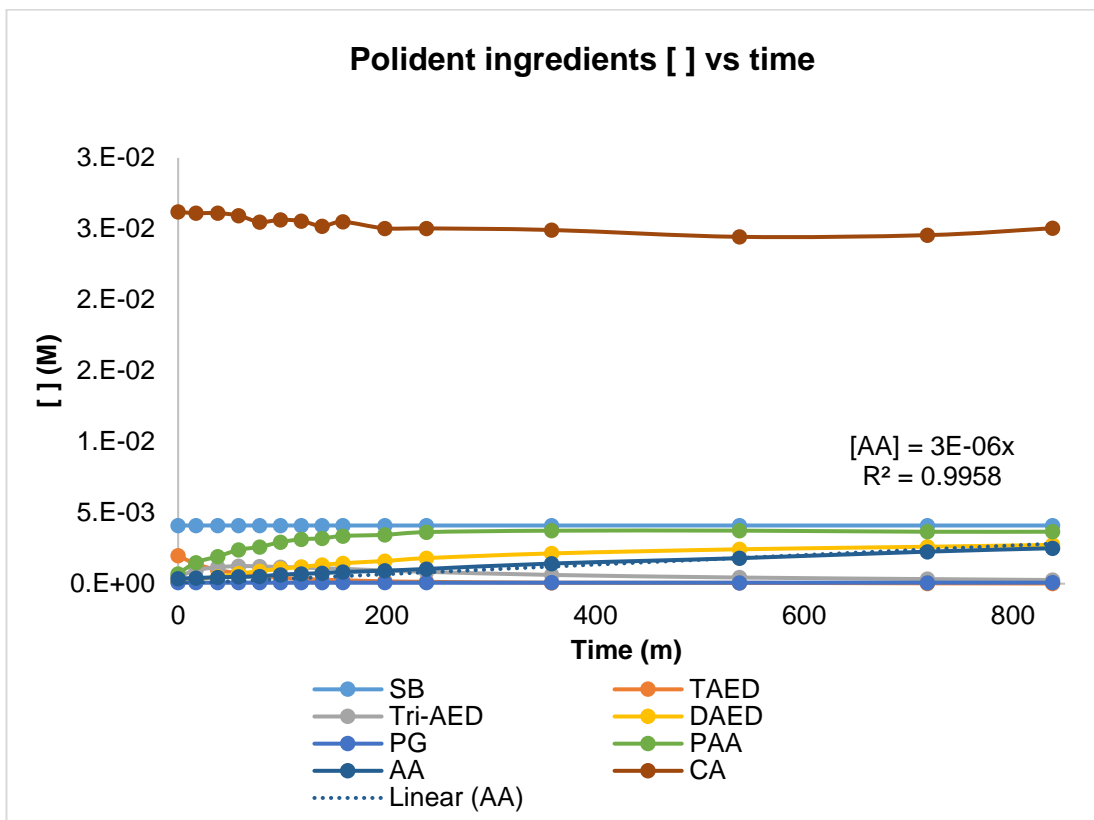


Figure 37 – Kinetic study of the Polident tablet dissolved at RT (approx. 22°C), sodium benzoate was used as reference concentration. The equation shown in the graph shows the acetic acid (AA) formation rate at 3 μM per minute.

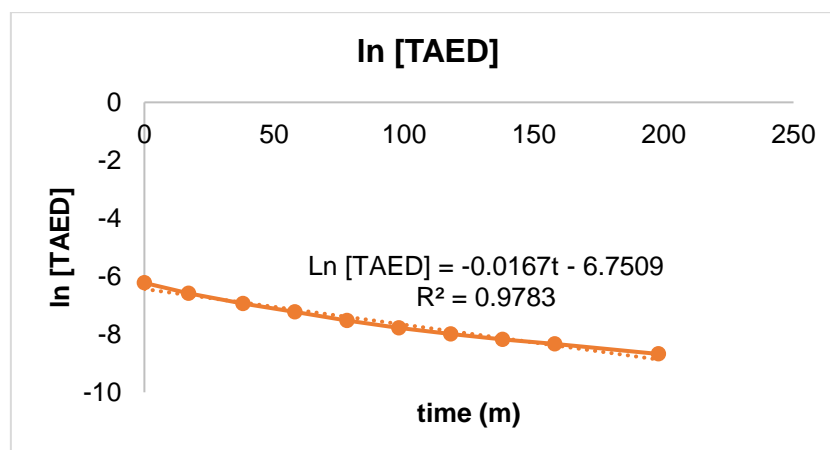


Figure 38 - Ln [TAED] vs time.

After plotting Ln [TAED] against time, Figure 38 was observed that the TAED perhydrolysis was a first order reaction.

Afterwards the effects of the temperature on the kinetics of the reaction were followed for the tablet decomposition at 40 °C and 60 °C and

the experimental results used to determine the kinetic laws that regulate our reaction are shown in the following page.

Figure 39 presents the plot of the Ln [PAA] at 60 °C against time. The TAED decomposition reaction is a first order reaction. A study made by Si *et al.* (2014)⁸⁷ also reported the same findings. Their research elucidated the bleaching mechanism involved in the generation of hydroxyl radical through reaction between TAED/H₂O₂ and showed that peracetic acid decomposition via hydrolysis and spontaneous decomposition follow first order kinetics while metal ion catalysed decomposition showed a second order mechanism⁸⁷. In our experiments the effects of metal ion catalysed decomposition were not observed due to the use of deionised water.

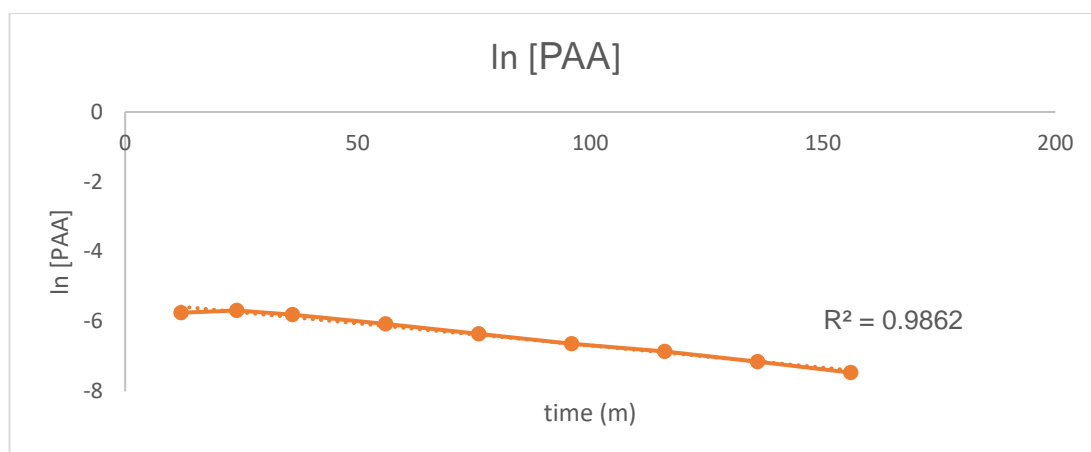


Figure 39 - Ln [PAA] vs time at 60 °C.

In Figure 40 are presented the effects of the temperature on the kinetics of the reaction, how the decomposition of TAED increased with the temperature as well the subsequent reactions leading to the production of peracetic acid and acetic acid. In all the experiments the total peracetic acid produced peaked at approximately 4 mM the temperature increase reduced the time to achieve that concentration, at 22 °C took nearly 6 hours, at 40 °C 3 hours and at 60 °C 24 minutes, the acetic acid formation rate also increased with the temperature from 3 $\mu\text{M}\cdot\text{m}^{-1}$ at 22°C to 10 $\mu\text{M}\cdot\text{m}^{-1}$ at 40°C and 70 $\mu\text{M}\cdot\text{m}^{-1}$ at 60 °C.

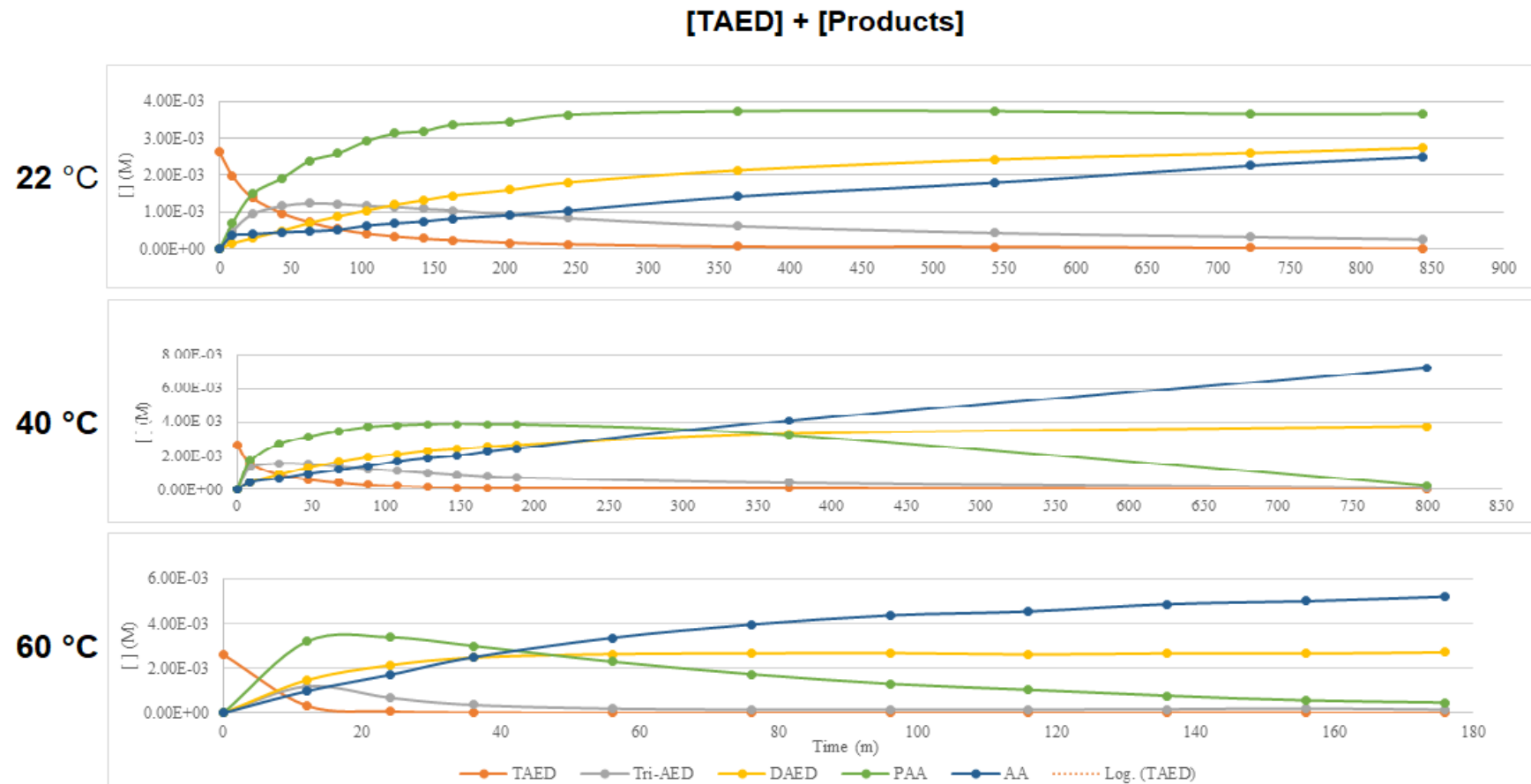


Figure 40 – Polident tablet decomposition kinetic study. The figure focus on the TAED perhydrolysis and its downstream of products (Tri-AED, DAED, peracetic acid (PAA), acetic acid (AA) at 22 °C, 40 °C and 60°C. (The time scale varies between experiments).

Table 10 - Maximum concentration of peracetic acid detected by NMR and the acetic acid formation rate for the initial period.

| | Max [PAA] mM | Time (m) | Acetic acid formation Rate |
|-------------|---------------------|-----------------|---------------------------------------|
| 22°C | 3.74 | 363 | $3.0 \times 10^{-6} \text{ M.m}^{-1}$ |
| 40°C | 3.88 | 148 | $1.0 \times 10^{-5} \text{ M.m}^{-1}$ |
| 60°C | 3.39 | 24 | $7.0 \times 10^{-5} \text{ M.m}^{-1}$ |

By applying the Arrhenius law to this study also allowed to calculate the activation energy¹¹⁹ of the conversion of peracetic acid into acetic acid and the DAED formation activation energy.

The Arrhenius law begins with the determination of the reaction rate, at constant temperature, that is given by the formula:

$$rate = k[A]^a[B]^b$$

Where: rate is the reaction rate in $\text{mol.dm}^{-3}.\text{s}^{-1}$, k is the rate constant in s^{-1} , $[A]$ and $[B]$ the concentration of the reactants in mol.dm^{-3} , a and b the order of the reaction in respect to A and B respectively. The rate constant (k) also corresponds to the slope of the [concentration] vs time

The rate equation determines how the reaction rate develops when the concentration of the reactants change following a rate constant that is dependent on temperature. While the Arrhenius law considers the changes in temperature and its effects on the reaction rate bringing into perspective the activation energy. The activation energy corresponds to the minimum energy necessary for the reaction to take place.

The Arrhenius law is described by the formula:

$$k = Ae^{-\left(\frac{Ea}{RT}\right)}$$

Where k is the rate constant at a specific temperature, A is the exponential factor or rate at which the collisions between the molecules take

place, E_a the activation energy ($\text{J}\cdot\text{mol}^{-1}$), R the gas constant $8.314 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ and T the temperature in Kelvin.

By applying the logarithm, we can transform the law into the logarithmic form

$$\ln K = \ln A - \left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right)$$

From the logarithmic form the slope of the resulting equation corresponds to $\frac{E_a}{R}$ therefore by plotting the experimental data as $\ln k$ vs $1/T$ it is possible to effectively determine the activation energy for each relevant step of the reaction taking place.

So, by applying the values experimentally found, the formation rates of DAED (Figure 41) that signals the TAED complete decomposition and the formation rates of acetic acid that corresponds to the degradation of the peracetic acid generated by the reaction, the activation energies were determined.

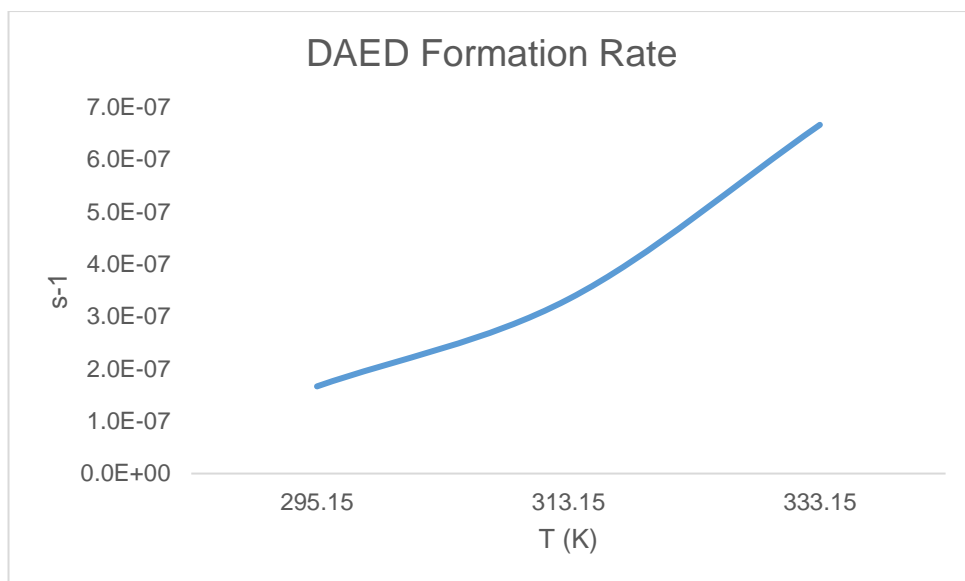


Figure 41 – DAED formation rate at different temperatures.

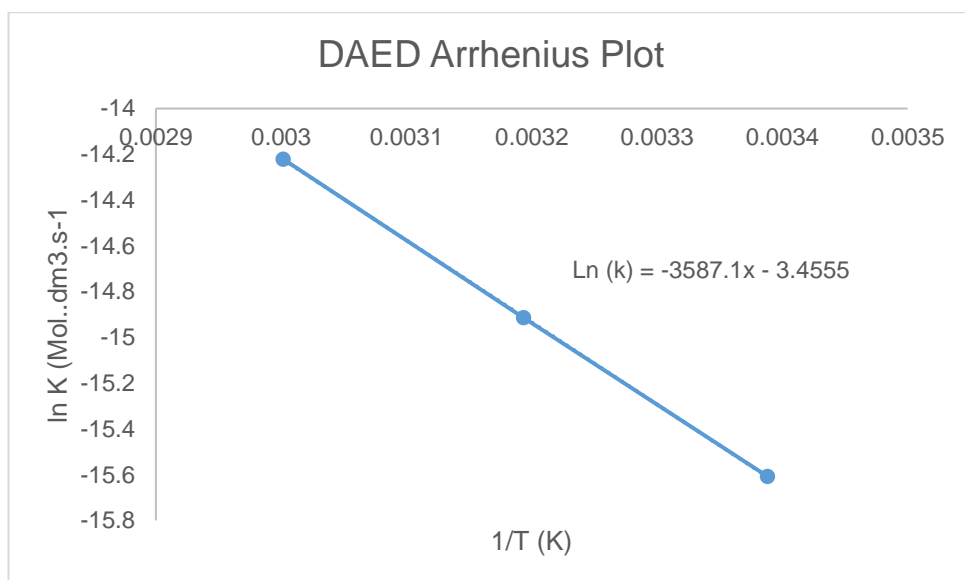


Figure 42 – DAED Arrhenius Plot. The slope of the chart will be used to determine the activation energy shown in Table 11.

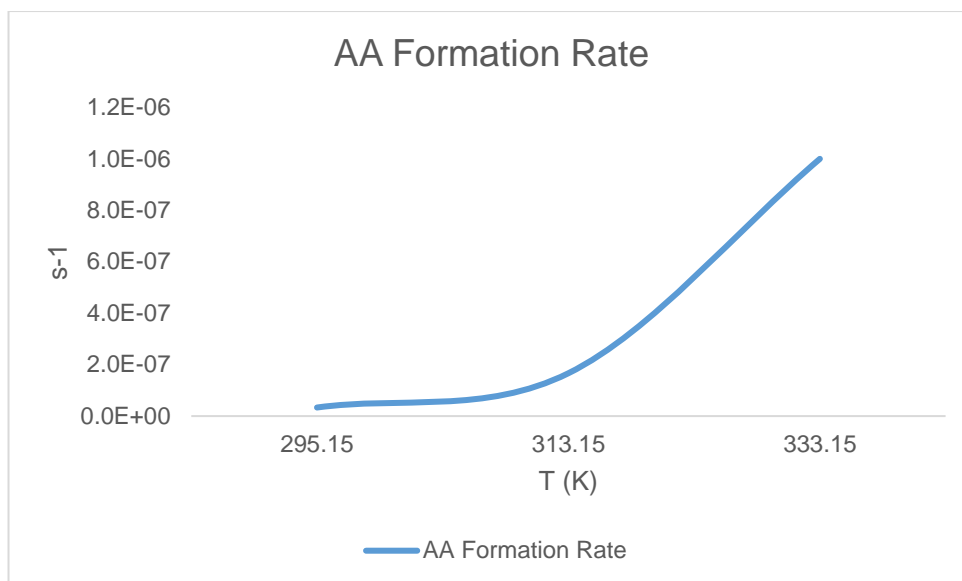


Figure 43 – Acetic acid formation rate at different temperatures.

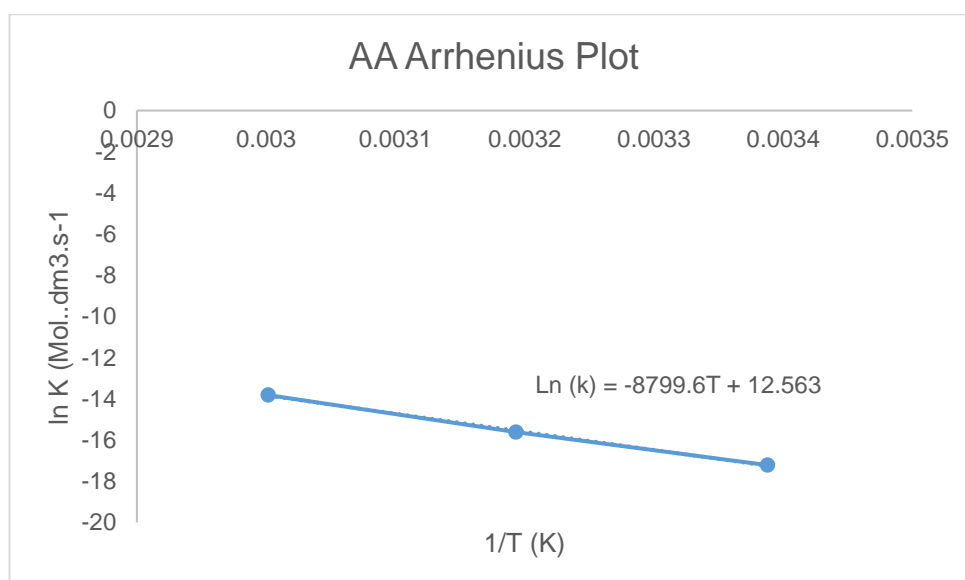


Figure 44 – DAED Arrhenius Plot. The slope of the chart will be used to determine the activation energy shown in Table 11.

The slope of the equation of the $\ln(k)$ vs $1/T$ is equal to E_a/R so by applying this rule was calculated the activation energies shown in the following table.

Table 11 – Arrhenius Law for DAED and AA formation and activation energies based on data extracted from Figures 42 and 44.

| | Arrhenius Law $\ln K = \ln A - \left(\frac{E_a}{R}\right)\left(\frac{1}{T}\right)$ | Activation Energy $Slope = \left(\frac{-E_a}{R}\right) \Leftrightarrow E_a = -Slope * R$ |
|------|---|--|
| DAED | $\ln (k) = -3587.1T - 3.4555$ | $E_a \text{ (DAED)} = 3587.1 * 8.314 \Leftrightarrow$ $E_a \text{ (DAED)} = 29823.149 \text{ J.mol}^{-1}$ |
| AA | $\ln (k) = -8799.6T - 12.563$ | $E_a \text{ (AA)} = 8799.6 * 8.314 \Leftrightarrow$ $E_a \text{ (AA)} = 73159.874 \text{ J.mol}^{-1}$ |

The TAED complete decomposition or DAED formation activation energy was 29.82 kJ.mol⁻¹ and for the AA activation energy was 73.16 kJ.mol⁻¹.

Considering that a single covalent bond has an energy between 150 – 400 kJ.mol⁻¹ and a hydrogen bond 2 – 161 kJ.mol⁻¹ ¹²⁰, it shows that the activation energy required for the reaction to take place will be easy to achieve.

After observing the kinetics and temperature effects on the Polident tablet during its decomposition, it was also relevant to observe the effects of the pH on the reaction kinetics. For this step were used the solutions CA100 and CA 200 with initial pH of 6.93±0.01 and 5.54±0.02 respectively. These solutions were studied following the same procedure as for the tablet at room temperature using quantitative ¹H-NMR and as expected only the TAED family (TAED, Tri-AED, and DAED), peracetic acid, acetic acid and citric acid, used to regulate the pH, showed up in the spectra. A kinetic study following the concentrations of the compounds along time is shown in Figure 45. For clarity the citric acid used as pH regulator and reference concentration was omitted from the graph.

The reaction between TAED and H₂O₂ also takes place but the kinetics of the reaction are affected. At a pH of 5.5 (CA200) showed the lowest acetic acid formation rate of 3.0 x 10⁻⁷ M.min⁻¹ while at the pH of 6.9 the acetic acid formation rate was 1.0 x 10⁻⁶ M.min⁻¹, the tablet acetic acid formation rate in this experiment was 2.0 x 10⁻⁶ M.min⁻¹. The acetic acid formation rate in the tablet varied slightly in comparison with the value from

the previous experiment this occurred because the experiment was conducted at room temperature. This difference can be attributed to minor variations in the temperature that can affect the reaction as rate and also some inaccuracies inherent to the manufacturing process of the tablet can affect the way that the tablet will perform.

The CA100 formulation was quicker in the generation of peracetic acid the maximum peracetic acid concentration was achieved after 20 min while for the CA200 and the tablet solution the concentration of peracetic acid was gradually building during the experiment. Despite the difference of pH between the tablet and the CA100 not being that different see Figure 45 a significant difference is observed in the development of the TAED perhydrolysis this may be due to the fact that the tablet has more ingredients thus reducing the reaction rate between TAED and H_2O_2 . On other perspective the low pH will not be ideal to promote the dissociation of H_2O_2 into HOO^- that is a crucial step in the perhydrolysis of TAED hence the CA200 solution showing a slower formation rate.

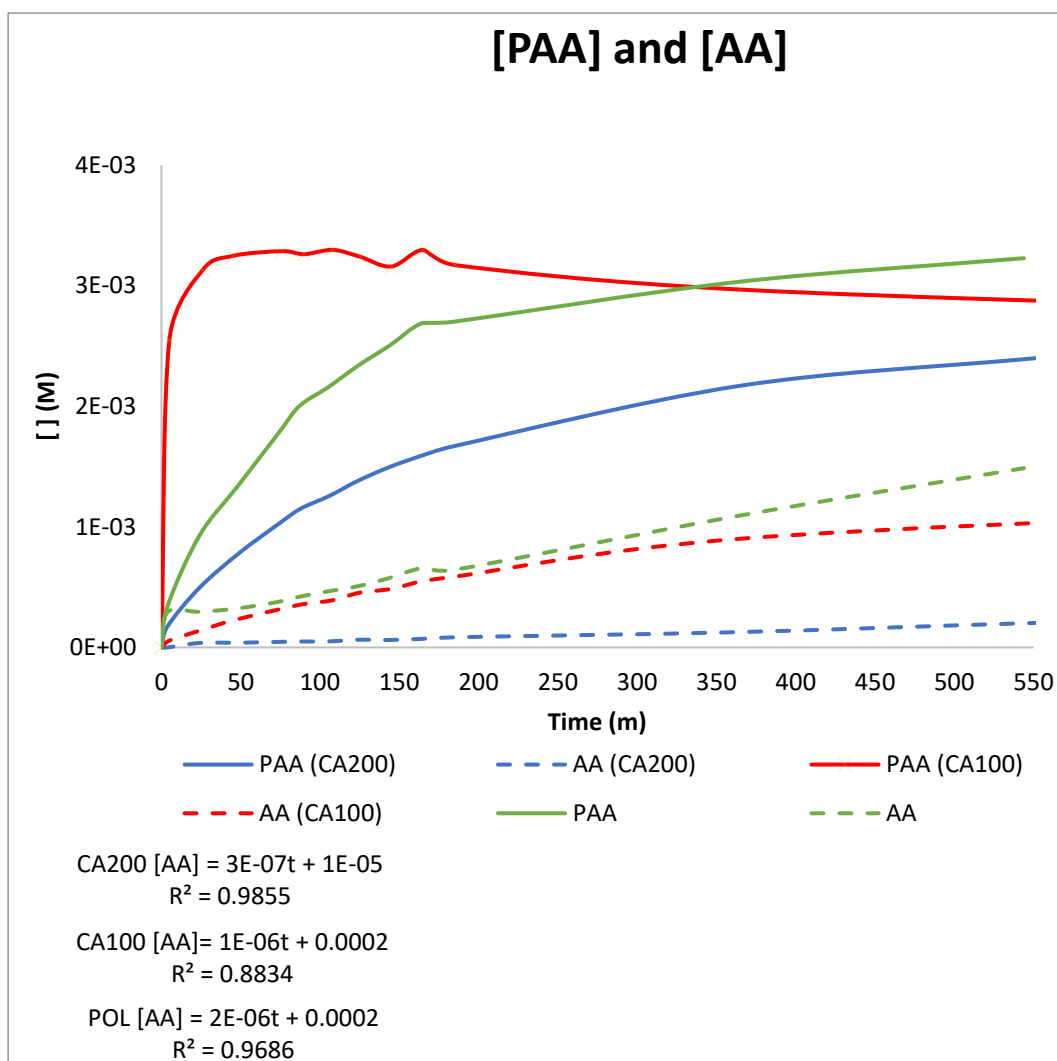


Figure 45 – TAED perhydrolysis products of the Polident tablet, CA100 and CA200 at RT. The green line shows the reaction taking place in the tablet mixture for comparison and the equation shown below the legend corresponds the AA formation by each formulation.

So far the results suggested that a higher pH will be beneficial for the perhydrolysis of TAED but must be taken into account that a high pH will not be beneficial for the generation of the desired final product, singlet oxygen. From the temperature point of view the higher the better but the final application for the product will limit this factor. In the case of dentures it is recommended to use warm water (~40 °C) only to protect the denture.

Despite being a promising result only the next round of experimental work aimed at the detection of singlet oxygen will be able to accurately determine the consequences of the variation on the pH.

3.2 Fluorometric Studies

3.2.1 Introduction

Due to the low concentrations of singlet oxygen (SO) generated within the TAED/NaPC system a direct detection method such the measurement of its infrared phosphorescence at 1270 nm was not feasible¹²¹. Therefore, after using ¹H-NMR to quantify the composition of the tablet and simplified formulations, a fluorimetry experiment using a fluorometric probe to detect reactive oxygen species (ROS) in the first stage of the experiments Dichlorodihydrofluorescein (DCFH) was used to detect the presence of ROS including the H₂O₂. In the final set of experiments Singlet Oxygen Sensor Green (SG) a fluorescent probe specific to the detection of singlet oxygen¹²² was used.

DCFH is a probe used to measure reactive oxygen species (H₂O₂, HO• and ROO•). It is an indirect method that relies on the oxidation of DCFH by reactive oxygen to a fluorescent compound DCF (Figure 46) with excitation maxima at 498 nm and emission at 522 nm.

DCFH can also be oxidised by enzymes such as catalase and superoxide dismutase and also compounds with high oxidation potential we need to also consider the existence of autoxidation. All together this can lead to an increase in duration of the fluorescence signal even after the production of ROS has stopped, resulting in some difficulties to effectively quantify the amount of ROS being generated^{123,124}.

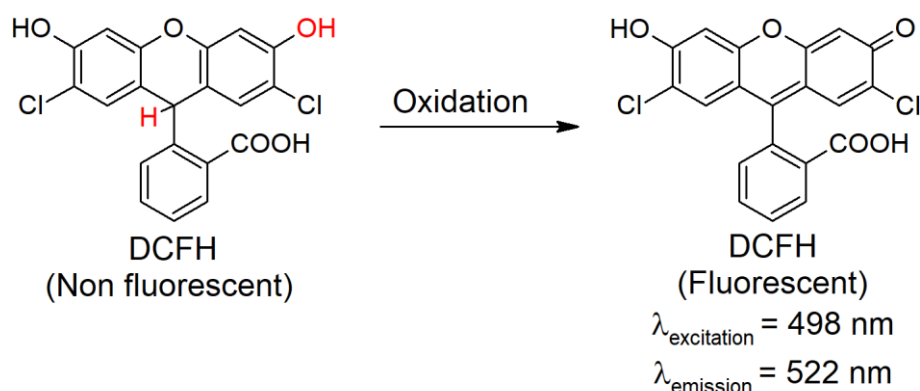


Figure 46 – Mechanism of DCFH conversion to DCF.

It is known that DCFH, has the capacity to be oxidised by a range of ROS¹²⁵ and this can be exploited to compare the difference in the amount of ROS generated in a solution despite the fact that this does not allow us to distinguish and quantify the ROS generation. For this experiment it will be useful to observe the contribution of H₂O₂ alone and if there is an increase in ROS after reaction with TAED.

On the other hand SG in a similar fashion to DCFH also reacts with a ROS but follows a different, slightly more complex mechanism to DCFH, making it specific to singlet oxygen detection¹²⁵.

The structure of SG has two parts: a trapping moiety, an anthracene derivative, and a fluorophore, the fluorescein moiety¹²⁶ shown in Figure 47.

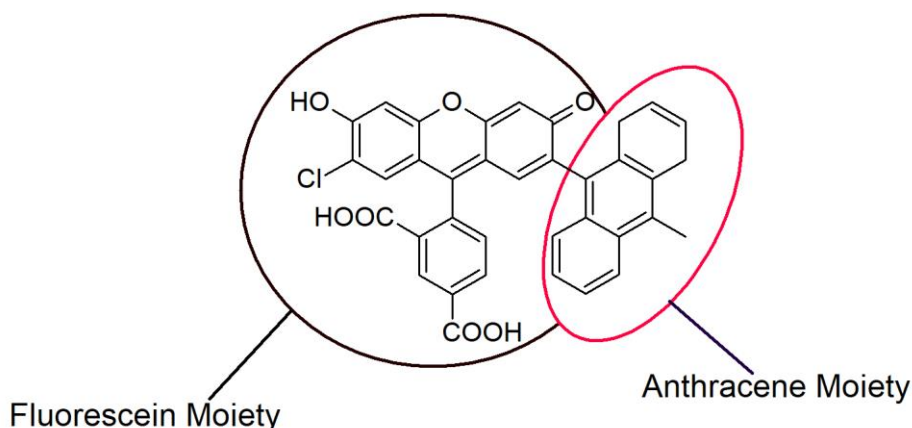


Figure 47 - Singlet oxygen sensor green molecule showing the fluorescein moiety responsible for the fluorescence and the anthracene moiety that traps and reacts with singlet oxygen.

The mechanism for detection and signalling relies on the fact that the anthracene part of the molecule is a strong electron donor and through photo-induced electron transfer will quench the fluorescein fluorescence. In the presence of singlet oxygen, the anthracene forms anthracene endoperoxide and since the highest occupied molecular orbital of anthracene endoperoxide has a lower energy in comparison with the fluorescein this results on the fluorophore becoming “active” when exposed to light^{122,126} Figure 48.

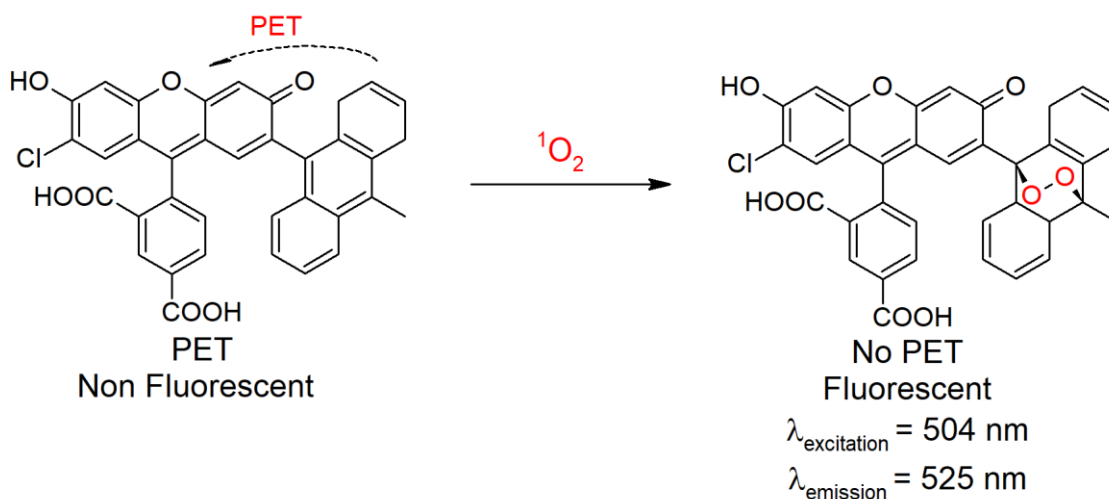


Figure 48 - Singlet oxygen sensor green fluorescence schematic. In the absence of photo-induced electron transfer the sensor green becomes photoactive¹²⁶.

SG is the favourite candidate used in the detection of singlet oxygen due to its specificity for this particular radical and the simplicity attached to the use of fluorescent probes in comparison with other methods¹²² hence, for the final studies using the simple formulations (TAED, NaPC and citric acid) Singlet Oxygen Sensor Green (SG) was used as to detect singlet oxygen generation.

For the initial part of the study a placebo tablet prepared by the Polident manufacturer with no actives (TAED, NaPC, potassium monopersulphate (MPS)), was used. To the placebo tablet, an aliquot of NaPC (at tablet concentration) was mixed and its fluorimetry measured, then TAED was added and mixed (at tablet concentration) and the fluorescence intensity was measured. The increase in signal showed that effectively more ROS were being produced and contributing for the oxidation of DCFH. The presence of MPS within the commercially available tablet was also considered, since this compound is known possess oxidizing properties. On the second part of the study singlet oxygen sensor green was employed to detect the presence of singlet oxygen in the samples and observe variations between the tested samples.

3.2.2 Materials and Methods

It is widely reported singlet oxygen shows an increased half-life in the deuterium oxide in comparison with that in water⁸¹. So, in order to observe that, in the first stage of this set of experiments we used DCFH and compared the intensity of the fluorescence signals between H₂O and D₂O as solvent. For the initial fluorimetry studies, solutions of TAED 0.021 g/ml (0.092 M), sodium percarbonate 0.051g/ml (0.325 M) respectively in D₂O and H₂O and a solution of DCFH 0.005 g/ml (10 mM) in ethanol were prepared.

Then using a Fluorimeter – SpectraMax M2. Excitation λ = 490 nm, Emission λ = 500 - 700 nm was measured. 1 ml of each of the prepared

solutions (TAED and NaPC) fluorescence was measured in the presence and absence of DCFH at 1 μ M concentration. Afterwards a fresh solution with equal volumes of TAED and NaPC were prepared and the DCFH fluorescence was measured as previously.

Following this first experiment as mentioned before, a commercially available Polident tablet was used alongside a placebo tablet, provided by GSK, with freshly prepared solutions of the active ingredients on its own in the same way as for the $^1\text{H-NMR}$ and pH studies in order to mimic the tablet concentrations using D_2O . See table below. Then the solutions shown in table 13, were prepared in duplicates in H_2O and D_2O and DCFH was employed. DCFH can be oxidised by several ROS¹²³.

The stock solutions shown in table 12 were prepared at 6X the original tablet concentration so upon mixture of all the components, in equal volumes, their final concentration will be equal to the tablet concentration, If all the components are not being used the remaining volume will be completed with D_2O and the NaPC aliquot is added in last prior to measuring the fluorescence, in order to delay the start of the main reaction.

Table 12 – Main tablet components stock solution prepared for each experiment. The solutions were prepared at 6X tablet concentration to account for the dilution that incurred from mixing different solutions.

| Ingredient | Stock solution (g/mL) |
|---------------------------------------|------------------------------|
| TAED | 3.59×10^{-3} |
| NaPC | 8.64×10^{-3} |
| Citric Acid | 2.16×10^{-2} |
| Potassium Monopersulfate (MPS) | 1.30×10^{-2} |
| Sodium bicarbonate | 4.13×10^{-2} |
| Sodium carbonate | 1.04×10^{-2} |

Table 13 – Tested solutions

| Stock | TAED | NaPC | MPS | Polident | Placebo | D ₂ O |
|-----------|--------------|------|-----|----------|---------|------------------|
| Condition | Volume in ml | | | | | |
| 1 | 0 | 0 | 0 | 0 | 0 | 5 |
| 2 | 0 | 0 | 0 | 5 | 0 | 0 |
| 3 | 0 | 0 | 0 | 0 | 5 | 0 |
| 4 | 1 | 1 | 0 | 0 | 4 | 0 |
| 5 | 0 | 0 | 1 | 0 | 5 | 0 |
| 6 | 1 | 0 | 1 | 0 | 4 | 0 |
| 7 | 0 | 1 | 1 | 0 | 4 | 0 |
| 8 | 1 | 1 | 1 | 0 | 3 | 0 |

For this part of the experiments the same spectrophotometer was used using an excitation wavelength of 490 nm, as before, but the detection wavelength was set to the maximum emission found in the previous experiment at 522 nm.

The tablet solutions were prepared by dissolving 0.11 g of crushed tablets in 6 ml D₂O. Then to the placebo tablet “the missing compounds” (TAED, NaPC and MPS) were added to try to mimic the original compounds at the tablet concentration and observe the contribution of each of the actives in the generation of ROS.

From this point forward we will be using a 96 well plate to increase the output of our results and decrease the variability within each experiment.

Therefore to 2 ml of the prepared solutions plus the Polident tablet, 2 ml solution, 2 µl of the DCFH at 10 mM was added and 200 µl of the resulting solutions were aliquoted into the wells of a 96 wells plate, incubated for 20 min at room temperature and its fluorescence was measured using the spectrophotometer plate reader function with the following settings, excitation at $\lambda = 490\text{nm}$ and emission detection at $\lambda = 522\text{ nm}$, as described before. To rule out auto fluorescence, from within the samples, all the samples were analysed with and in the absence of the probe.

Table 14 - 96 well plate loading scheme.

| | No DCFH | | | | | | 5 μ M DCFH | | | | | |
|---|-----------------------------|---|---|---|---|---|-----------------------------|---|---|----|----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | D ₂ O | | | | | | D ₂ O | | | | | |
| B | Polident | | | | | | Polident | | | | | |
| C | Placebo | | | | | | Placebo | | | | | |
| D | Placebo + TAED + NaPC | | | | | | Placebo + TAED + NaPC | | | | | |
| E | Placebo + MPS | | | | | | Placebo + MPS | | | | | |
| F | Placebo + MPS + TAED | | | | | | Placebo + MPS + TAED | | | | | |
| G | Placebo + MPS + NaPC | | | | | | Placebo + MPS + NaPC | | | | | |
| H | Placebo + MPS + TAED + NaPC | | | | | | Placebo + MPS + TAED + NaPC | | | | | |

After finding out that MPS was interfering with our probe signal it was decided to run a separate experiment to assess the development of the DCFH signal throughout time to compare the tablet and the placebo tablet in the presence of NaPC and TAED. This experiment followed the same methods as the experiment described before with the difference that the MPS solution was not added to any of the mixtures and was the only missing compound in the placebo + TAED + NaPC solution.

On the final set of experiments, it was followed a similar approach only changing the fluorescent probe from DCFH to SG and using the novel formulations used for the ¹H-NMR studies, the CA100 and CA200 tested alongside the Polident tablet and the placebo tablet. 2 μ l of a 5 mM SG in methanol (prepared as described by the manufacturer) was added to the relevant test wells of the 96 well plate. Fluorescence was measured at 1 minute intervals for 180 minutes.

It is also important to mention that this last experiment was performed using a different fluorimeter, a Fluoroskan Ascent, Labsystems due to a change of facility. This instrument had fixed wavelengths settings and, the experiment was conducted using an excitation wavelength of 485 nm and detected an emission wavelength of 538 nm. This instrument also enabled to perform a kinetic measurement following the development of the fluorescent signal through time.

3.2.3 Fluorimetry Studies Results

In this instance it was observed the generation of, other reactive oxygen species (ROS) than H_2O_2 , resulting from the reaction between TAED and NaPC and, at the same time ascertain that by using D_2O we can effectively increase the lifetime of singlet oxygen in solution and increase the intensity of the fluorescent signal generated by the probe being used.

The solutions were prepared TAED and NaPC to be used separately, and then a solution of TAED and NaPC mimicking tablet concentrations. Afterwards its fluorescence was measured with and without the probe alongside with controls of solvent solutions.

In the Figure 49 was observed that upon excitation with 490 nm the probe produced fluorescent maximum emission at 522 nm and was consistent throughout all the tested samples. Only the samples containing NaPC had shown an increase in fluorescence. The NaPC solution (dark green) and the NaPC/TAED solution (red), signal was greatly increased in comparison with NaPC alone. Considering that the NaPC solution offers H_2O_2 to the reaction mixture we were expecting to detect this signal.

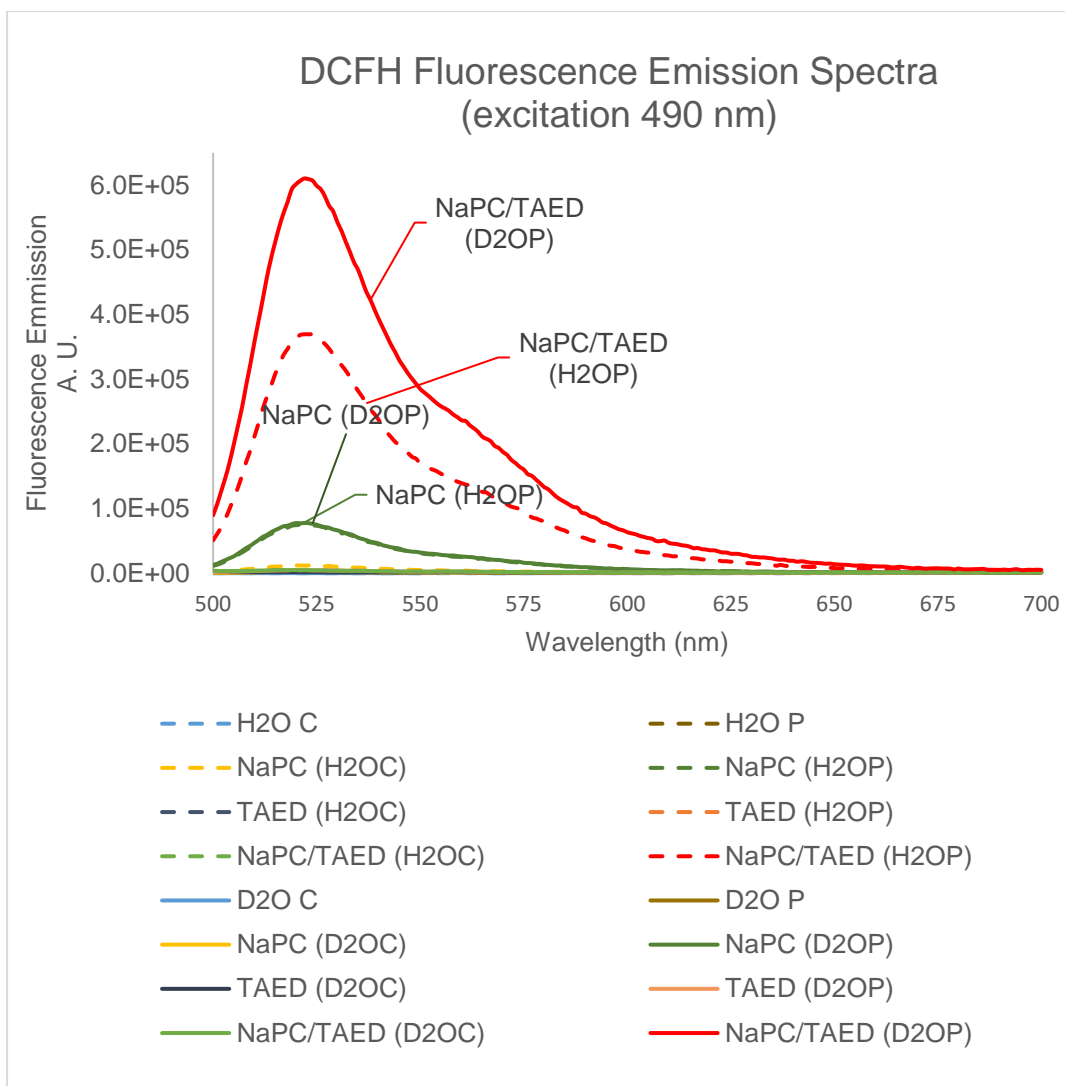


Figure 49 -- DCFH emission spectra of the solutions prepared. The full line corresponds to the samples prepared with D2O while the dashed line to samples prepared with H2O. The use of D2O only increased the fluorescence signal arising from the NaPC/TAED samples.

Furthermore, by adding TAED the signal increased meaning that a reaction is taking place and increasing the type of ROS present in solution.

By comparing the full line vs the dashed line, the difference in fluorescence signal between H₂O and D₂O is evident. By following the green line can be observed that there was no difference in the signals emitted by the NaPC solution in H₂O or D₂O while for the NaPC/TAED solution an increase in the signal was noted, that corroborates with the findings reported in the literature that associate the increased rate of oxygen consumption in

deuterium oxide with the presence of singlet oxygen^{81,126,127}. Thus, the increase of the signal observed between the solutions of NaPC/TAED elucidates the generation of singlet oxygen.

Satisfied with the first confirmation of the presence of singlet oxygen it was needed to observe the same effect but in this instance within a formulation. This part of the study had the objective to detect the generation of further ROS other than the existent H₂O₂ given by NaPC within the Polident formulation using the placebo tablet. A placebo tablet was provided by the Polident manufacturer produced under the same specifications without the main actives, TAED, NaPC and potassium monopersulfate (MPS). MPS is used in the tablet classified as antimicrobial agent and is widely reported as a strong oxidiser used to clean the water in swimming pools and water treatment plants^{128–130}.

From the results obtained (Figure 50) was observed that MPS interacts with the DCFH used to detect the fluorescence. The DCFH molecule is oxidised by ROS to produce the fluorescent DCF but it can also be oxidised by strong oxidisers¹²⁵. From the results it was observed that the placebo tablet solution alone does not emit any fluorescence but this can be changed by adding MPS or NaPC. The addition of TAED on its own did not increase the fluorescence signal.

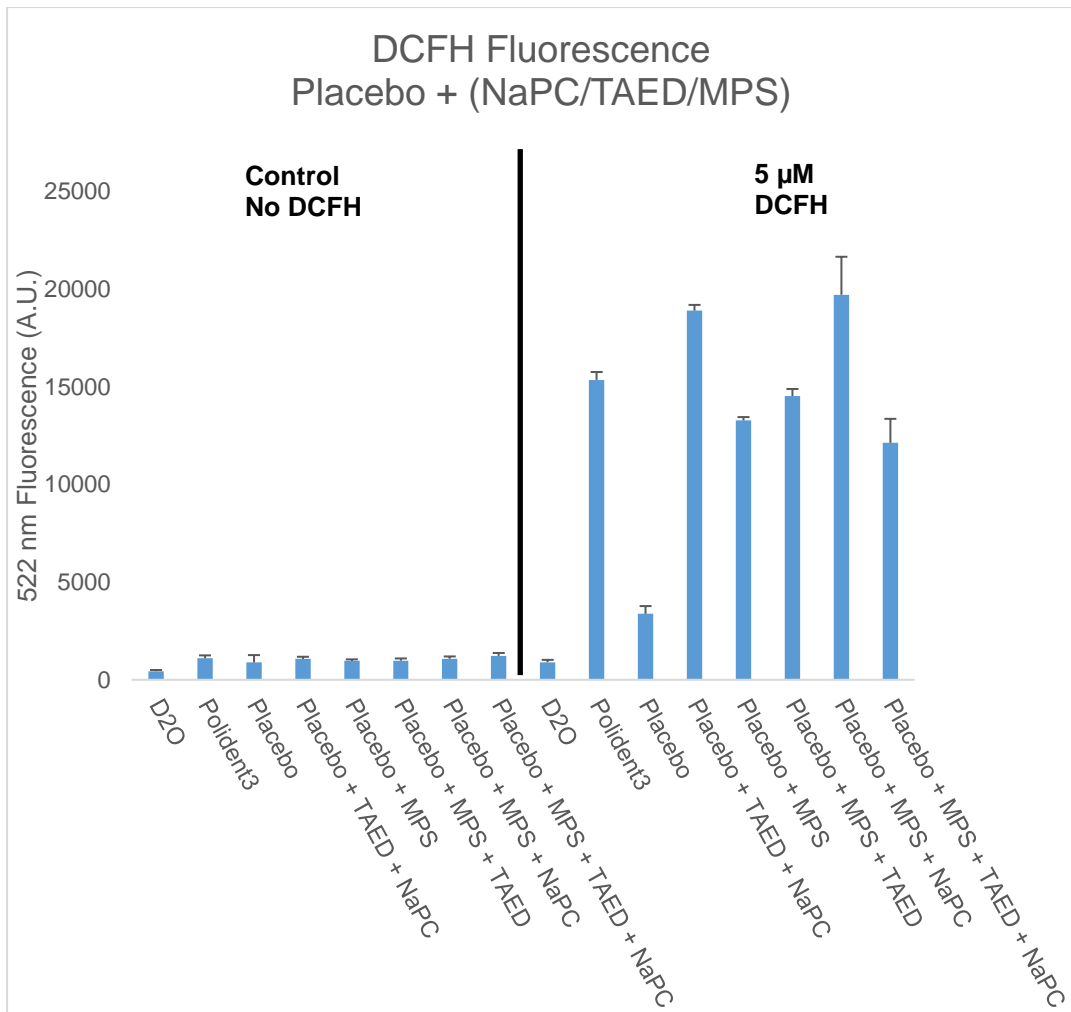


Figure 50 - DCFH fluorescence of Polident and Placebo tablet on its own and with the addition MPS, NaPC and TAED.

Since the DCFH was generating a signal in the presence of MPS Figures 50 and 51, it was decided to remove it from the kinetic study using the fluorescent probe, further the curve shaped signal shows that the DCFH is being rapidly consumed or quenched due to this additional reaction between MPS and DCFH. To observe this effect the experiment was conducted with and without the addition of MPS shown in Figure 51.

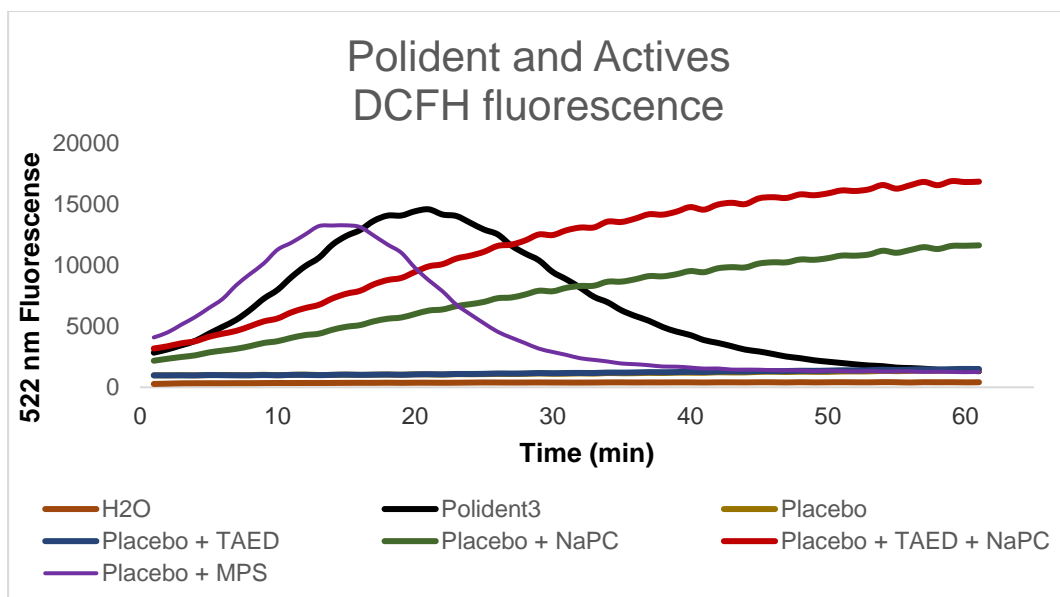


Figure 51 - DCFH fluorescence time development using a Polident solution alongside the placebo tablet with TAED, NaPC and MPS.

The figure above show the results of the fluorimetric study using Polident and different combinations of ingredients added to the placebo tablet, the red line shows the solution with the placebo tablet with TAED and NaPC at polident tablet concentrations and it was noted a steady increase in fluorescence, leading to the conclusion that there was a gradual generation of ROS as shown by the NMR experiments.

By adding NaPC to the placebo solution (green line) there is in an increase in the fluorescence signal in comparisson with the placebo tablet with TAED, or on its own. Neither of the latter showed any detectable fluorescence signal. As noted in the previous experiment the addition of MPS originated a signal probably due to oxidation of the probe by MPS. This experiment showed that TAED and NaPC when used in combination are an effective way to further generate other ROS in addition to the H₂O₂ provided by NaPC alone.

After completing the studies using DCFH it was decided to use a probe specific to detect our final product singlet oxygen. In this instance the Polident tablet was once more used as reference alongside the placebo tablet without any additions and the two new simplified formulations CA100 (pH 6-7) and CA200 (pH 5-6) were also tested, Figure 52.

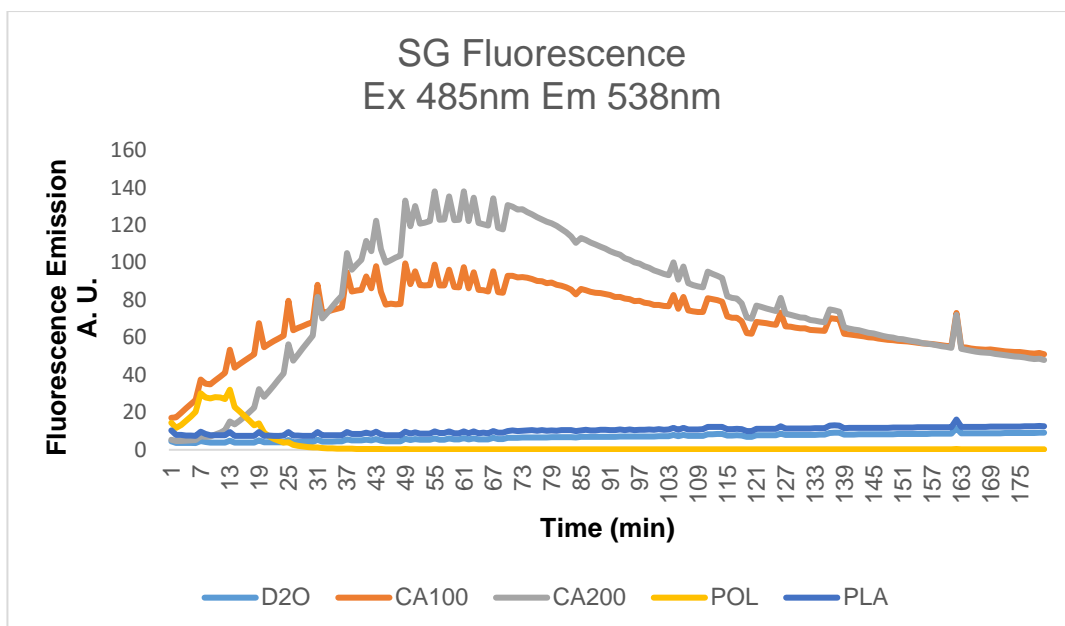


Figure 52 - Time development study of the fluorescence of Singlet Oxygen Sensor green in the presence of the Polident tablet, placebo tablet, CA100 and CA200 formulations.

Interestingly, this final study showed that the pH was very important in the stability and lifetime of the singlet oxygen generated. Both novel “formulations”, CA100 – citric acid + TAED + NaPC, pH 7-8 and CA200 – citric acid + TAED + NaPC, pH 5-6, generated a higher fluorescence signal than the polident tablet. By comparing the fluorescence signals between CA100 and CA200 it was observed that after 30 minutes the signal of CA200 become higher than the CA100 leading to the conclusion that a lower pH may impair the initial decomposition of hydrogen peroxide (H_2O_2) into perhydroxil anion ($-OOH$) but on other hand contributes for the ideal spontaneous decomposition of peracetic acid into acetic acid and singlet oxygen. The 1H -NMR studies shown in Figure 45 (Page 88) show that in the CA100 formulation the generation of peracetic acid was the fastest from all formulations a result that agrees with a fast decomposition of H_2O_2 therefore a rapid perhydrolysis of TAED and subsequent generation of peracetic acid occurs at a faster rate. While the lower pH CA200 was the slowest in generating peracetic acid.

3.3 Antimicrobial Studies

3.3.1 Introduction

Building upon the findings of the previous experiments, ¹H-NMR and fluorometric studies proved that TAED and NaPC were in fact reacting to produce peracetic acid (¹H-NMR) and that subsequently would lead to the production of a strong reactive oxygen species (ROS) and singlet oxygen. We proceeded to the final stage of this project where some of the antimicrobial properties of Polident and of the novel formulations, CA100 and CA200 were observed, namely, the minimum inhibitory concentration (MIC), that as the name implies stand for the minimum concentration of an agent where organism growth is inhibited; minimum bactericidal concentration (MBC), the minimum concentration of an agent required to kill the test organism, both of these tests are done for a 24 hour period; and the antimicrobial efficacy (kill time) that determines how many organism are killed in a specific period of time.

The tests mentioned above were performed using microorganism suspensions of *Streptococcus mutans*, *Candida albicans* and *Fusobacterium nucleatum*. The organisms chosen are representative of normal oral commensal organisms in this instance *Streptococcus mutans*, a Gram-positive coccus an early colonizer of the mouth cavity^{106,107}, associated with caries¹³¹, an increase in the population of *S. mutans* is also observed in denture wearers¹³², and recent studies show a link between the pathogenicity of *S. mutans* colonizing dentures in carcinogenesis¹³¹. *Fusobacterium nucleatum*, a Gram-negative rod an intermediate colonizer important in the establishment and development of the oral biofilm and plays a significant role in periodontal disease^{133–135}, plus a yeast, *Candida albicans*, a constituent of the oral microbiome⁷ known to be a common opportunistic pathogen associated with denture stomatitis^{19,109}, oral thrush²², as well a wide range of systemic complications¹³³. The bacterium *F. nucleatum* was only tested against Polident and placebo tablet despite of the fact of having an important role in biofilm development, this bacterium is a obligate anaerobe and

struggles to survive for long periods when exposed to oxygen¹³⁴. The test solution produces reactive oxygen species thus, making this organism a particularly easy target. During biofilm development *F. nucleatum* requires an established biofilm and the presence of other organisms to attach⁶. Therefore, in order to grow a biofilm with *F. nucleatum* another biofilm model would need to be developed greatly increasing the costs and time in for the overall study.

In the MIC experiment the highest concentration of Polident to be tested will be 50% of the tablet contents, due to assay limitations. Since a commercially available product is being used it is only possible to work with the final concentrations of the product. The test methodology recommends preparing the test formulations at higher concentration to account for these dilutions¹³⁶.

In the kill time assay the highest tablet concentration that can be used was 80% of the tablet solution concentration, due to assay limitations. In the kill time tablets solutions were inoculated with the microorganisms to observe the efficacy of the tablet against planktonic microorganisms. The findings of these experiments gave an initial screening of the antimicrobial efficacy of the Polident tablet. The MIC/MBC of the individual tablet components was also tested to ascertain which of the ingredients possessed antimicrobial properties.

Then following these initial studies, a Calgary Biofilm device⁹⁸ also known as MBEC device was employed to develop single species biofilms of *S. mutans* and *C. albicans* to determine the minimum biofilm eradication concentration (MBEC). This was done by measuring the viability of the remaining biofilm after exposure to the antimicrobial solutions, the 96 identical biofilms generated provide an excellent screening tool to simultaneously compare the efficacy of a range of formulations.

At the same time, a dual species biofilm grown from the same organisms on a PMMA disk was also tested and the remaining biofilm was visualized using scanning laser confocal microscopy (SLCM) and scanning

electron microscopy (SEM). In this part of the study Polident was used alongside the placebo tablet, with no actives and MPS, as comparative references to the developed formulations CA100 and CA200.

Following that, the Calgary biofilm device (Figures 53 and 54), was tested and validated for its suitability to develop the required biofilms. Two types of MBEC devices were used, one uncoated (polystyrene) and one coated with hydroxyapatite used to mimic oral cavity conditions by simulating the teeth material. The MBEC device permitted to grow several individual identical biofilms, alongside that experiment, PMMA disks, were also inoculated to be used for imaging techniques scanning laser confocal microscopy (SLCM) and scanning electron microscopy (SEM) (Figure 55). In this initial test the inoculate was serially diluted to find a suitable inoculum concentration to grow the required biofilm. Once this was found, the biofilm was produced and challenged using different test conditions and their respective controls moving towards a more accurate result of biofilm removal efficacy.



Figure 53 - Calgary biofilm device – Lid with pegs, where biofilm grows, and the base, a 96 well plate.

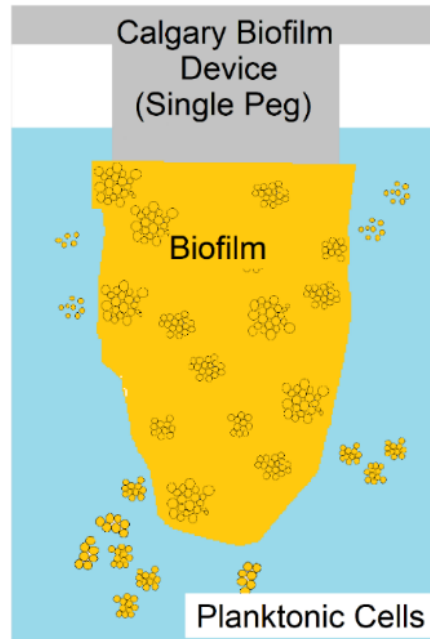


Figure 54 – Biofilm growth on the MBEC device pegs.

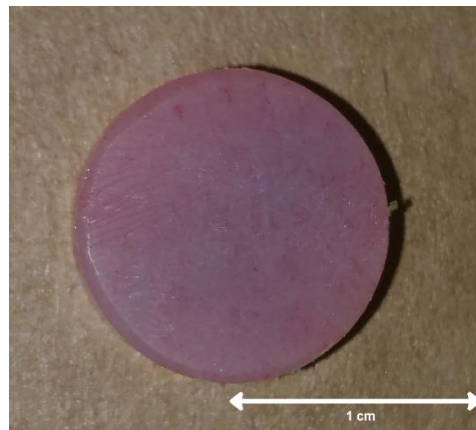


Figure 55 – Lucitone PMMA disk 1.2 cm diameter and 0.4 cm depth.

Then after challenge the biofilm viability was measured using the AlamarBlue cell Viability Assay. This assay is quite robust and has been in use for more than 50 years to measure the viability of a wide range of animal, bacterial and fungal cells. This is a non-invasive assay that allows the viability of the cells to be measured without disrupting the biofilm, it relies on the reduction of resazurin (dark blue/violet, non-fluorescent) to resofurin (pink, fluorescent) ^{137–139}, see Figures 56 and 57. The colour changes in the media can be observed at naked eye but a fluorimeter can give a better insight and help to understand minor variations between the tested conditions by measuring the

resofurin fluorescence an excitation wavelength of 530-560 nm and emission detection at 590 nm¹⁴⁰.

The reduction of resazurin occurs as a result of cell metabolism but it is not known if it occurs in the cytoplasm membrane or in the circulating media as a chemical reaction but it is widely accepted that it is related to oxygen consumption through metabolism¹⁴¹.

Since this method is quite simple and does not require the disruption of the biofilm it allows a quick and easily measure of biofilm viability in several identical biofilms simultaneously and compares the removal of biofilm achieved by each of the testing solutions¹⁴¹.

On a parallel study using the same test solutions the biofilms grown on the PMMA disks were stained using live and dead stain. This technique is a technique that enables visualization of formed biofilms with minimal disruption by staining the live cells in green and dead cells in red (Figure 58). A commercially available product LIVE/DEAD BacLight Bacterial Viability Kit consisting of two fluorescent dyes SYTO 9 (green) and propidium iodide (PI) red was used. Both are nuclear stains that bind in a non-specific way to DNA but PI can only bind to the DNA of dead cells or cell with compromised membrane integrity while SYTO 9 can target both live and dead cells^{142,143}.

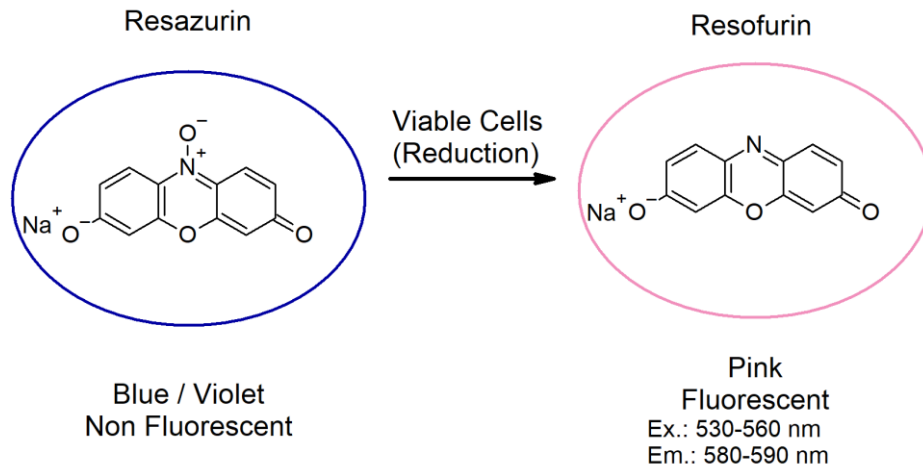


Figure 56 – AlamarBlue, the blue compound resazurin is shown on the left and the pink compound resulting from the reduction by living cells is shown on the right-hand side.

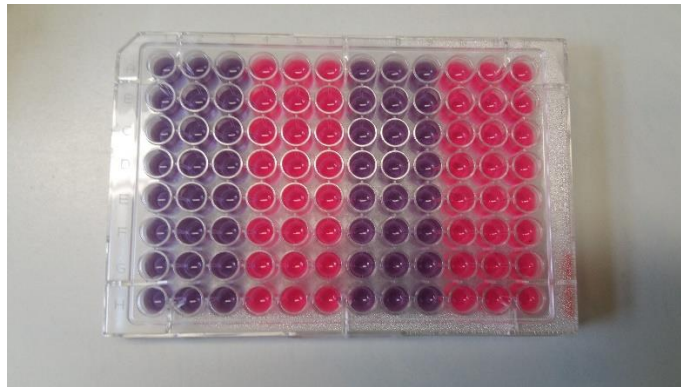


Figure 57 – AlamarBlue after incubation with *C. albicans* as test microorganism and without microorganism In the blue/violet wells show AlamarBlue resazurin and pinkish resofurin that results from the reduction of the resazurin into resofurin.

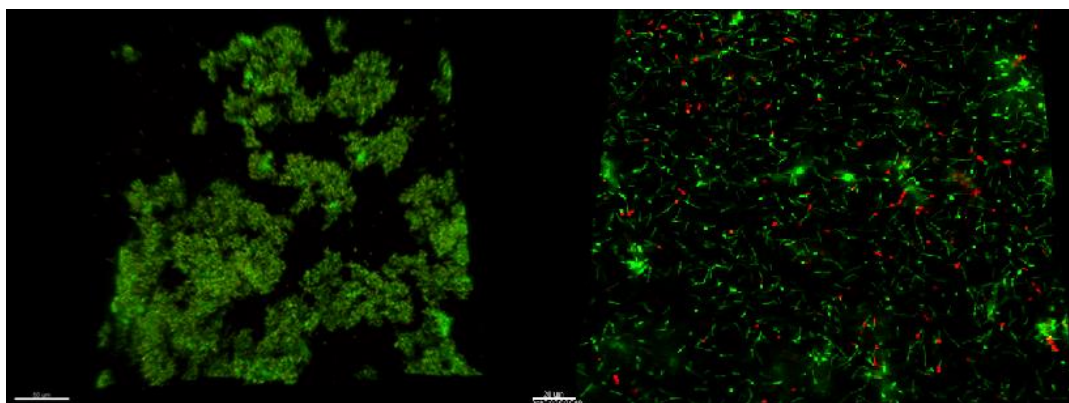


Figure 58 – Example of scanning laser confocal microscopy images. Left *S. mutans* 48 hours biofilm grown on a hydroxyapatite disk. Right 60 X magnification of *Bacillus subtilis* suspension stained with BacLight Bacterial Viability Kit, the cells stained in green represent the live cells, while the ones in red dead cells.

PI exhibits enhanced fluorescence when bound to DNA, by intercalating into DNA every four to five base pairs¹⁴⁴. SYTO 9 in a similar fashion is also a DNA intercalator and both compete for the same DNA spots but, since PI has a higher affinity towards DNA, when both are present PI, can displace SYTO 9 taking its place^{144,145}, therefore making cells with defective membranes fluoresce in red (dead) and cells with intact membranes fluoresce in green (live).

Alongside the SLCM specimens, the remaining set of disks were fixed and prepared to be observed using SEM. Since an electron beam is used to image the sample this technique provides a higher resolution than visual microscopy techniques that relies on the use of visible light to image the specimen^{146,147}. SEM allows observation of the removal of biofilm and eventual damage after treatment with the solutions containing ROS.

3.3.2 Material and Methods

3.3.2.1 Antimicrobial Efficacy (Kill Time)

In this test all the three organisms selected, *S. mutans* UA159, *C. albicans* 3153A and *F. nucleatum* NCTC10562 were challenged by Polident tablet and placebo for a specific period of time (1, 3 and 10 minutes) according to the guidelines established by the British Standard - EN1276 - Chemical disinfectants and antiseptics — Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas — Test method and requirements¹³⁶.

Due to the addition of the inocula that is in suspension the test material was diluted, therefore the Polident and placebo tablets could only be tested at an 80% of the concentration at which they were manufactured. For the kill time determination, the following reagents and equipment were used: 96 deep well plate, sterile dH₂O; Columbia blood agar (Oxoid, Hampshire, UK) (Columbia Agar + 5% Horse Blood); as test agents Polident and placebo tablet (no actives); Tryptone Soya Broth (Oxoid, Hampshire, UK) was used to neutralize the test solutions and to dilute the test suspension, phosphate buffer saline solution (PBS) (Oxoid, Hampshire, UK) was used to prepare the inocula and further dilute the test suspension; a spectrophotometer; Buffered Peptone water (Oxoid Hampshire) a media that provides the ideal conditions for the recovery of cells (peptone 10g/l, sodium chloride 5.0 g/l, disodium phosphate 3.5 g/l, potassium dihydrogen phosphate 1.5 g/l, pH 7.2 ± 0.2). Ultrospec 2000, Pharmacia Biotech was used to measure the bacterial suspension turbidity at 600 nm against a blank of sterile suspension medium.

All organisms were stored at -80 °C, and fresh plates prepared and stored as required. Propagation of the test organisms *Streptococcus mutans* UA159, *Candida albicans* 3153A, *Fusobacterium nucleatum* NCTC10562. *S. mutans* and *C. albicans* were grown for 24 hours at 37°C in 5% CO₂ on Columbia horse blood agar, while *F. nucleatum* was grown for 48 hours at

37°C in an anaerobic incubator (Ruskinn Concept Plus) with an atmosphere of 80% N₂, 10% CO₂ and 10% H₂ also using Columbia horse blood agar. These fresh cultures grown, after the incubation period, were kept at 4 °C for up to two weeks. Once established the cultures would be checked visually by gram staining.

The inocula were made by harvesting several loopful of colonies from the culture plates mentioned above and dilute in PBS to a concentration of 10⁷⁻⁸ CFU/mL (Abs_{550nm} *F. nucleatum* = 0.3-0.5, *S. mutans* = 0.3-0.5, *C. albicans* = 1.0-1.3) and then further diluted 1:100, 100 ml of this suspension was added to 19.9 ml of double strength brain heart infusion (DSBHI) to achieve inocula of around 10⁵⁻⁶ CFU / mL. This was later confirmed by plating serial dilutions (1:10) of the inocula in Columbia blood agar (see appendix for agar preparation) and incubate under the required growth conditions for the tested organism. For a simplified version see Figures 59 and 60.

Propagation of microbial culture

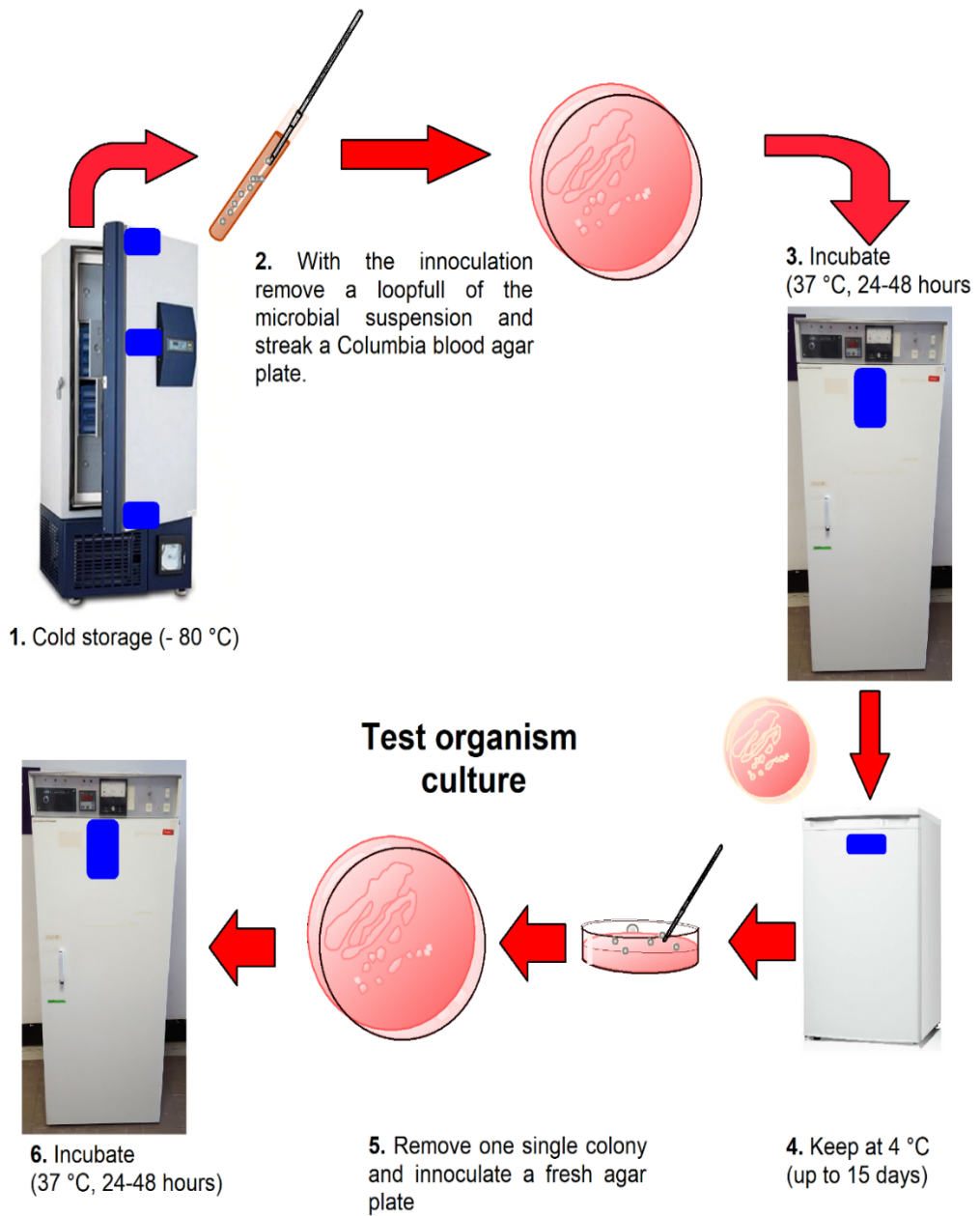


Figure 59 – Microbial culture propagation and maintenance method schematic.

Inocula Preparation

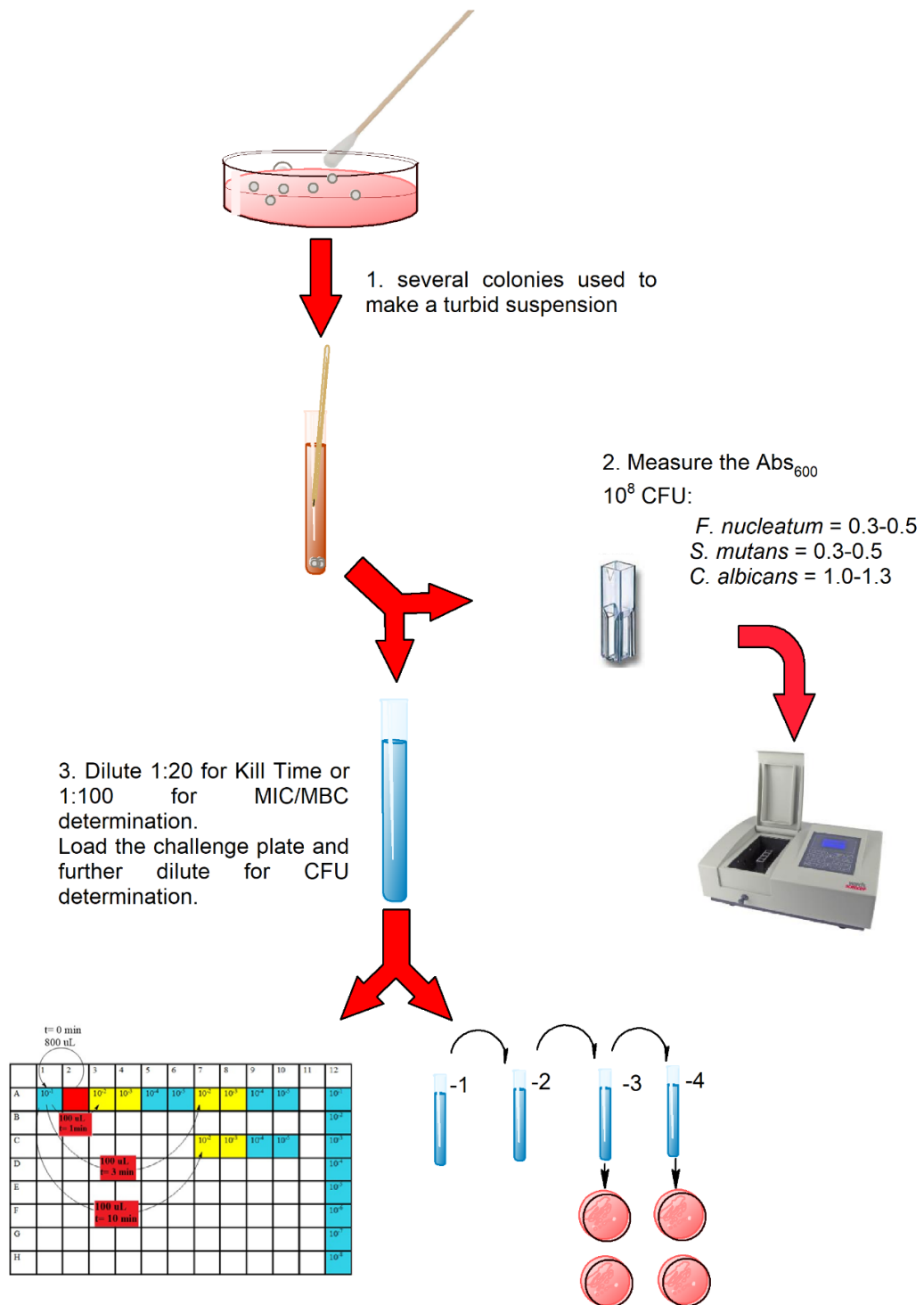


Figure 60 – Schematic of the inocula preparation. After making a turbid suspension the absorbance at 600 nm was measured to estimate the CFU/ml of the suspension. When the measurements for each organism are at the required interval (*F. nucleatum* and *S. mutans* 0.3, *C. albicans* 1.0 – 1.3) the suspension is further diluted 1:100 in PBS to reach the inocula concentration around 10⁵⁻⁶ CFU/ml.

The following volumes were added to the wells: to the well A1 100 µl of peptone water were added, to well A2 900 µl of testing agent (Polident tablet, placebo tablet) to the wells A3, A4, A7, A8, C7 and C8 900 µl of tryptone soya broth were added, To the wells A5, A6, A9, A10, C9 and C10, 900 µl of peptone water and to the wells A12 to H12 900 µl of peptone water were added. See the Figure 61 below.

| Solution/suspension | Well | Volume (µL) |
|---------------------|--------------------------|-------------|
| Test | A2 | 900 |
| PBS | A12 – H12 | 900 |
| | A5, A6, A9, A10, C9, C10 | 100 |
| TSB | A3, A4, A7, A8, C7, C8 | 900 |
| Inocula | A1 and A12 | 100 |

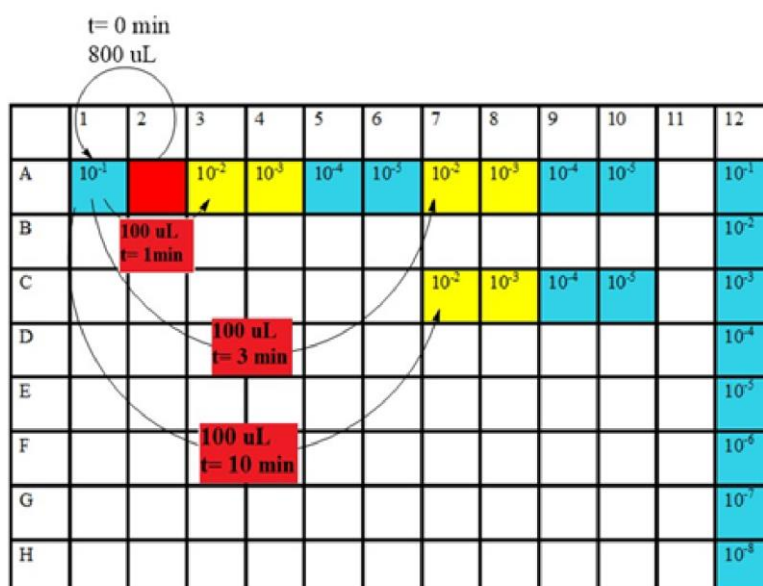


Figure 61 - Schematic of the 96 well plate load out for the experiment, first the solutions and inocula (on top table) were prepared, then the volumes added to the corresponding wells in a 96 well plate. The arrows indicate the time of the testing suspension transfer to the neutralizing solution. Following that there is a 10 minute step for neutralization of the testing solutions.

Following the initial loading of the 96 well plate the inoculum was prepared as shown in Figure 60.

Then 100 μ l of the inocula were added to the well A1 and mixed thoroughly. Then 800 μ l from well A2 was added and mixed with the contents of well A1 and the timer was started.

To determine the viable count at time zero 100 μ l of the inoculum was also transferred to well A12, mixed and serially diluted from A12 to H12 and 100 μ l of each well was plated in duplicate on the relevant growth agar used to grow the organism being tested.

After 1, 3 and 10 minutes 100 μ l were removed from well A1 and transferred to well A3, A7 and C7 respectively. After mixing, the contents of the wells were incubated for 10 minutes at room temperature to neutralize the antimicrobials. This step is essential to stop the effects of the antimicrobial agents, and to ensure that the antimicrobial activity is stopped after the exposure time. Following the 10 minutes neutralization step 100 μ l was transferred from well A3 and serially diluted (1:10) in wells A4, A5, and A6. For the 1 minute step, from A7, after the 10 minutes neutralization step, 100 μ l were transferred and serially diluted on wells A8 to A10 and finally the same was followed for the well C7 and diluted in the wells from C8 to C10.

Finally, 100 μ l were removed from each well (A3 to A6, A7 to A10 and C7 to C10) and plated on Columbia blood agar using 3 mm glass beads (in duplicates) and all the plates were incubated under the required conditions.

After incubation, the colonies were counted, and the results compared with the viable count at time zero (initial) and the percentage of reduction was calculated. This experiment was repeated 3 times for each microorganism and solution tested and the results are the average of the colony counts of each determination.

3.3.2.2 Minimum Inhibitory Concentration / Minimum Bactericidal Concentration (MIC / MBC)

For the MIC double strength (DS) brain heart infusion broth (Oxoid, Hampshire, UK), deionised H₂O (autoclaved 121°C for 15 minutes), Columbia blood agar (Columbia agar (Oxoid, Hampshire, UK) + 5 % horse blood (Oxoid, Hampshire, UK); as test agents Polident and placebo tablet (no actives) dissolved as per manufacturer indications, one tablet in 150 ml of H₂O, 40 °C; and the solutions shown in the tables below:

Table 15 – Stock solutions. A stock solution at 6X the tablet concentration for each main ingredient (TAED, NaPC, CA, MPS, Na₂CO₃, NaHCO₃) to account for the dilution resulting from mixing all together. Since for the MIC/MBC experiment a 2X concentration is required the final test solution corresponds to 2X the tablet concentration.

| Solution | Tablet concentration in 150 ml (g/mL) | Test Concentration [2X tablet] | Stock Concentration [6X tablet] |
|--------------------------------|---------------------------------------|--------------------------------|---------------------------------|
| TAED | 5.99×10^{-4} | 1.2×10^{-3} | 7.2×10^{-3} |
| NaPC | 1.44×10^{-3} | 2.88×10^{-3} | 1.7×10^{-2} |
| Citric Acid | 3.60×10^{-3} | 7.2×10^{-3} | 4.3×10^{-2} |
| Potassium Monopersulfate (MPS) | 2.16×10^{-3} | 4.32×10^{-3} | 2.6×10^{-2} |
| Sodium bicarbonate | 6.88×10^{-3} | 1.38×10^{-2} | 8.3×10^{-2} |
| Sodium carbonate | 1.73×10^{-3} | 3.46×10^{-3} | 2.08×10^{-2} |

Table 16 - Combined Polident ingredients tested solutions. When not all the 5 ingredients were used 1 ml of H₂O was added to the solution to replace the missing ingredient.

| Stock Condition | TAED | NaPC | CA | MPS | NaHCO ₃ | Na ₂ CO ₃ | H ₂ O |
|-----------------|------|------|----|-----|--------------------|---------------------------------|------------------|
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 2 | 1 | 1 | 0 | 0 | 0 | 1 | 3 |
| 3 | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| 4 | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| 5 | 1 | 1 | 0 | 1 | 0 | 0 | 3 |
| 6 | 1 | 1 | 1 | 0 | 0 | 0 | 3 |

The solutions shown in the previous table correspond to the same ones used for the kinetic studies and pH studies at a higher concentration to account for the method dilution.

Test organisms *Streptococcus mutans* UA159, *Candida albicans* 3153A, came from fresh cultures grown in Columbia blood agar and kept at 4 °C for up to two weeks. *Fusobacterium nucleatum* NCTC10562 was grown in Columbia blood agar and kept in the Anaerobic incubator and propagated every week up to 4 times.. Prior the experiment one colony was removed with a inoculation loop and a fresh Columbia blood agar inoculated and incubated as follows: *S. mutans* and *C. albicans* were grown for 24 hours at 37°C in 5% CO₂ on Columbia horse blood agar; *F. nucleatum* was grown for 48 hours at 37°C in an anaerobic incubator (Ruskinn Concept Plus) with an atmosphere of 80% N₂, 10% CO₂ and 10% H₂ using Columbia horse blood agar.

Phosphate Buffered Saline solution (PBS) (Oxoid, Hampshire, UK) was used to prepare the inocula and a spectrophotometer was used to measure the bacterial suspension turbidity.

The inocula were made by harvesting several loopful of colonies from the plates mentioned above and diluted in PBS to a concentration of 10⁷⁻⁸ CFU/mL (Abs_{550nm} *F. nucleatum* = 0.3-0.5, *S. mutans* = 0.3-0.5, *C. albicans* = 1.0-1.3) and then further diluted 1:20, 1 ml of this suspension was added to 19 ml of double strength BH broth I to achieve inocula of around 10⁶⁻⁷ CFU / mL. This was later confirmed by plating serial dilutions (1:10) of the inocula in Columbia blood agar and incubate under the required growth conditions for the tested organism. The organism *F. nucleatum* was only used in the initial testing against the Polident and placebo.

Then 100 µL of sterile dH₂O was then added to the wells in columns 2-12, rows A, B, C and all wells in rows G and H of a 96-well microtitre plate. 100 µL of DS BHI broth were also added to the wells in row F. Following that 100 µL of the test solution were added in to the 1-2 wells of rows A, B and C (triplicate) and two-fold serially diluted along the rows from column 2 onwards

for rows A, B and C. Since the challenged organism is in suspension, the addition of the organism to the test solution will result in a decrease in the starting concentration of the test solution, this is normally accounted for by preparing the test solution at double of the required test concentration. In the case of a manufactured product we cannot avoid this in testing therefore for the tablets (Polident and placebo) the initial tested concentration was at 50% of the total tablet concentration, see the following Figure 62.

| % [test] | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | 0.20 | 0.09 | 0.05 |
|--------------------------|--|----|------|------|------|------|------|------|------|------|------|------|
| % [Polident] or Placebo] | 50 | 25 | 12.5 | 6.25 | 3.13 | 1.56 | 0.78 | 0.39 | 0.20 | 0.09 | 0.05 | 0.02 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | (Organism) | | | | | | | | | | | |
| B | (Organism) | | | | | | | | | | | |
| C | (Organism) | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | Ve-1 (Test agent + DS BHI) | | | | | | | | | | | |
| G | Ve-2 (H ₂ O + DS BHI) | | | | | | | | | | | |
| H | Ve+ (H ₂ O + organism in DS BHI) | | | | | | | | | | | |

Figure 62 - 96 well plate loading schematic. Rows A, B and C test solution; row F negative control (*Ve-1*) broth and test agent; row G negative control (*Ve-2*) with water and DS BHI; and row H a positive control (*Ve+*) maximum growth of the organism in the absence of test solution. The % on top represent the total contents of testing solution.

Afterwards 100 µL of the inoculum suspension was added to the test wells, rows A, B, C and H. Also, alongside the test wells the following controls were set:

- 100 µL inoculum/broth + 100 µL sterile water (test organism viability) in row H (*Ve+*).
- 100 µL broth + 100 µL sterile water in row G (*Ve-2*).
- 100 µL test compound + 100 µL double strength broth in row F (*Ve-1*).

Making the experimental setting as shown in Figure 62 rows A, B and C test solution; row F negative control (*Ve-1*) to rule out false positives arising from reactions between broth and test agent; row G negative control (*Ve-2*) with water and DS BHI to rule out false positives arising from reactions between broth and water; and row H a positive control (*Ve+*) to establish maximum growth of the organism in the absence of the agent.

The 96 well plates were then incubated at 37°C, in a shaking incubator at 150 rpm under the required the growth condition used for each organism specified as above.

The MIC was read by measuring the Abs_{550nm} of the plates and was determined as the lowest concentration to show an absence of growth by comparison with the negative control.

For the MBC determination 10 μ l were removed from the wells containing higher concentrations of the test compound than the MIC (no growth detected) and plated on Columbia blood agar, then incubated under the required conditions. The MBC concentration is the lowest concentration at which no growth was observed.

3.3.2.3 Biofilm Studies

The biofilm studies were made by adapting the method developed by Konrat *et al.*, 2016¹⁴⁸ and the Innovotech MBEC Assay® with some modifications described below. The material used was brain heart infusion broth (Oxoid, Hampshire, UK), dH₂O (autoclaved 121°C for 15 minutes), artificial saliva (see appendix Section E for preparation) blood agar Columbia agar + horse blood (Original) (Oxoid, Hampshire, UK), Sabouraud dextrose agar (Oxoid, Hampshire, UK); as test agents Polident and a placebo tablet (no actives) were used; neutralization agent used was sodium sulphite (Na₂SO₃) (Sigma); test organism *Candida albicans* 3153A, was grown for 24 hours at 32°C in 5% CO₂ on blood SDA. Biofilm substrate: MBEC™ device (innovotech, Canada); Lucitone PMMA disks (RD128-Lucitone, Biosurface Technology Corporation) AlamarBlue® cell viability reagent. Fluorimeter (Fluoroskan Ascent – Labsystems. For the visualization experiments: Confocal microscopy Live/Dead® viability stain (LIVE/DEAD BacLight; Invitrogen, Paisley, UK) was used to stain the cells after the experiment and were then visualized using a scanning laser confocal microscope BioRad Radiance2100 (Zeiss, Welwyn Garden City, Herts, UK) and its software for development of the images. Scanning Electron Microscopy: 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) was used as a fixation buffer, grading ethanol solutions (50%, 70%, 90%, 100%), hexamethyldisilazane (Sigma-Aldrich), to dry the samples. Polaron E5000 SputterCoater (Quorum Technologies, Laughton, UK) and imaged using a scanning electron microscope XL30 field emission SEM (Philips, Amsterdam, Netherlands).

3.3.2.3.1 Inoculum preparation

The inoculum was prepared by inoculating 10 ml of sterile BHI with a single colony of the test organism followed by incubation for 18 hours (200 RPM, 37 °C). The organism was then collected by centrifugation (20 °C, 5000 RPM, 5 minutes) and washed twice using PBS and resuspended in fresh

artificial saliva to the required concentration. In each experiment, the inocula was confirmed by plating serial dilutions on Columbia blood agar in duplicate for viability counts.

The first stage of this set of experiments had the purpose to test the suitability of the MBEC™ device and PMMA disks to grow the biofilms and of the viability of the method using AlamarBlue. The organism *C. albicans* was used.

3.3.2.3.2 Determination of optimal cell density for the biofilm studies

To the first column/row of a 96 well plate 200 µL of the inoculum serially diluted 1:10 in BHI (1:2 in a parallel experiment). On the last row/column of the plate only BHI was used as a negative control to measure AlamarBlue fluorescence on its own and detect any interference with the media. Then add 20 µL of the stock AlamarBlue solution to achieve a 10% AlamarBlue concentration per well.

The plate was placed in the fluorimeter set for a kinetic measurement every 30 minutes with the following parameters excitation: 530 nm, emission 590 nm with shaking (200 RPM) at a temperature of 37 °C. This experiment proved that AlamarBlue was sensitive and robust enough for the set of experiments.

Following that the inocula was prepared in the same way as for the previous experiments but using artificial saliva (AS) as inoculation media. The wells of a 96 well plate was loaded with 150 µL the inocula and serially diluted 1:10 from row 12 – 4 and to rows 1 - 3 were loaded only with AS for a no growth control. This validation step was done using HA coated and uncoated MBEC devices to assess which would be more suitable for our experiments. For the PMMA disks a 24 well plate and 1.2 mL were used per

well with two disks on each. On this case only three 1:10 dilutions were made. The plates were then placed inside a humidity chamber and incubated for 24 hours (37 °C, 120 RPM).

Succeeding that the lids and disks were removed from the solution and washed with PBS for 30 seconds (96 wells plate 200 µL 24 well plate 2 mL) using a rotating shaker at 80 RPM. Afterwards a fresh 10% AlamarBlue solution in BHI was prepared 200 µL and 2 mL were loaded into a 96 and 24 well plate respectively and the MBEC™ device lid and PMMA disks were placed inside on the 96 well plate and 24 well plate respectively and incubated for 4 hours (120 RPM, 37 °C). Subsequently the lid and disks containing the biofilms were removed from the plates and the fluorescence of the remaining solution of BHI+ AlamarBlue was read using an excitation light of 530 nm and emission 590 nm to determine the viability of the biofilms. It is important to mention that the media used to measure the viability is not the same used throughout the experiment. The media required by the AlamarBlue manufacturer is BHI, this is a rich nutrient media and will not affect the growth of the organisms. Note that BHI is also the media used to propagate the organism in other steps.

After finding the ideal inocula concentration at 10^7 - 10^8 CFU/mL for *C. albicans*, the same concentration was used with *S. mutans*, the same procedure was followed to grow the biofilms to be challenge by the test solutions. 150 µl of the inoculum were added to some of the wells of a 96 well plate while the remaining wells were filled with 150 µL of fresh uninoculated AS for negative controls. The MBEC™ lid placed on top and incubated for 24 hours (120 RPM, 37 °C) inside a humidity chamber to prevent drying.

3.3.2.3.3 Biofilm Challenge using MBEC and PMMA disks

After the incubation period the MBEC™ was washed by immersion in PBS and gentle shaking 80 RPM for 1 minute at room temperature, to remove loose biofilm. At the same time tablets and test formulations were completely dissolved in dH₂O at 40 °C and then loaded into a challenge plate as shown in the Figure 63, in the following page, the control wells were loaded with 200 µL of dH₂O in column 1, Polident tablet solution in column 2 and placebo tablet solution in column 3. The test wells were, as well loaded, 200 µL of dH₂O in columns 4 - 6, Polident tablet solution in columns 7 - 9 and placebo tablet solution in columns 10 – 12.

In a similar fashion the biofilm grown on the PMMA disks as described before but on this instance after being challenged and neutralized from the 2 disks per well one was placed in the staining solution made of 1 µl PI (red) 2 µl syto9 (LIVE/DEAD BacLight Bacterial Viability Kit) and incubated in the dark at room temperature for 30 minutes; and the other placed in the fixative solution, an adaptation of the Karnovsky fixative (see annexes for description and preparation) and stored overnight at 4°C.

Since this was building upon the previous results, for the MBEC determination the test solutions used were only the Placebo, Polident and CA 200. Polident and Placebo tablets were diluted down to 25% while the CA200 formulation was diluted to 25%.

a)

| | Ve- Control | | | Ve+ Control | | | Test 1 | | | Test 2 | | |
|---|------------------|--------|--------|--------------------------------------|---|---|--------------------------------------|---|---|--------------------------------------|----|----|
| | H ₂ O | Test 1 | Test 2 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | No organism | | | Inoculated 10 ⁷ CFU/mL | | | Inoculated 10 ⁷ CFU/mL | | | Inoculated 10 ⁷ CFU/mL | | |
| B | | | | | | | | | | | | |
| C | | | | | | | | | | | | |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

b)

| | | Ve + Control | Test 1 | Test 2 | | Ve - Control |
|---|---|--------------------------------------|--------------------------------------|--------------------------------------|---|--------------|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| A | | | | | | |
| B | | Inoculated 10 ⁷ CFU/mL | Inoculated 10 ⁷ CFU/mL | Inoculated 10 ⁷ CFU/mL | | No organism |
| C | | Inoculated 10 ⁷ CFU/mL | Inoculated 10 ⁷ CFU/mL | Inoculated 10 ⁷ CFU/mL | | No organism |
| D | | | | | | |

Figure 63 – a) Loading diagram of the 96 well plate used to challenge the biofilm. Columns 4 – 12 are the pegs were the pegs were biofilm grown and 1 – 3 do not have biofilm attached to it. The test column contents were changed as required for each experiment. b) Loading diagram for the 24 well plate the wells B6 and C6 were loaded with AS and no organism and the wells B2-4 and C2-4 were inoculated with the same inocula as the 96 well plate. Two PMMA disks were then loaded in each well.

Afterwards the biofilm pegs were placed on the test plate (Figure 63 a)) and incubated for 10 minutes at room temperature with no agitation, then biofilm pegs were subsequently washed and neutralized in freshly prepared PBS + 0.5% Na₂SO₃ after treatment for a further 10 minutes and washed once more in PBS.

The lid was then placed on another 96 wells plate with a 10% AlamarBlue solution, freshly prepared, and incubated for 4 hours (37 °C, 120

RPM) and the AlamarBlue fluorescence was read (excitation 530 nm, emission 590 nm).

Then the data was normalized using the values obtained from the control wells *ve-* (negative control) H₂O (no growth) and *ve+* (positive control) H₂O maximum growth and converted into percentage of viable biofilm, all the experiments were repeated at least three times and the results were presented with the standard deviation.

3.3.2.3.4 Biofilm Visualization

The PMMA disks to be observed using CLSM were then removed from the staining solution, after 30 minutes and gently washed in PBS and placed in a fresh PBS and taken to be visualized using a the CLSM imaged with a 60x water immersion lens using a laser light source. Representative sections were imaged with a pixel definition of 1024 x 1020 pixels with no zoom and ImageJ 1.8.0 was used to process the images.

The SEM samples were removed from 4 °C and washed with 0.1M cacodylate buffer for 10 minutes (80 RPM) then transferred to the 50% ethanol solution for 10 minutes (80 RPM), this step was repeated for 70% ethanol, 90% ethanol and thrice for 100% ethanol. Then was placed on a solution of ethanol/HDMS (50:50) for 5 minutes after that a subsequent 5 minutes in 100% HDMS and air dried inside a safety cabinet.

Then the samples were sputter coated with 95% gold and 5% palladium using the SputterCoater and taken to the SEM to be visualized at 5 kV images were taken from representative sections at 1000X and 5000X magnification.

3.3.3 Results / Discussion

3.3.3.1 Kill Time

The kill time experiment, showed that *C. albicans* was a much more resistant organism than the bacteria used. One minute challenge by the Polident and placebo tablet were sufficient to eliminate *S. mutans* (Figure 64) and *F. nucleatum* (Figure 65). *C. albicans* was only successfully eliminated after a 10 minute exposure to the polident solution. Figure 66 shows that a time response occurred in the elimination of *C. albicans*, 1 minute 53% reduction, 3 minutes 79% reduction and 10 minutes 99.99%.

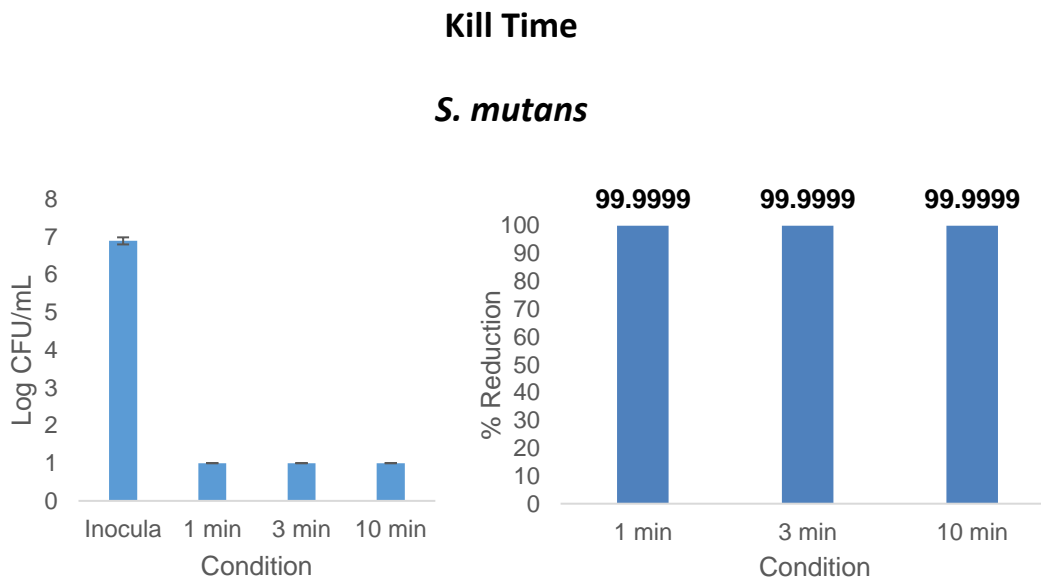


Figure 64 – Kill time results of Polident against *S. mutans*, the figure on the left shows the Log of CFU after the challenge and the right shows the percentage of reduction in the organism viability, the error bars show the standard deviation of three separate experiments.

From the kill time experiments with *S. mutans* and *F. nucleatum* Figures 64 and 65, no colonies grew on the plates of the undiluted

suspension after challenge with Polident showing that Polident is effective in killing those organisms after only one minute. With *C. albicans*, Figure 62, as literature suggests this tend to be a more resistant pathogen and its capability of having two diferent morphological forms seems to play to its advantage.

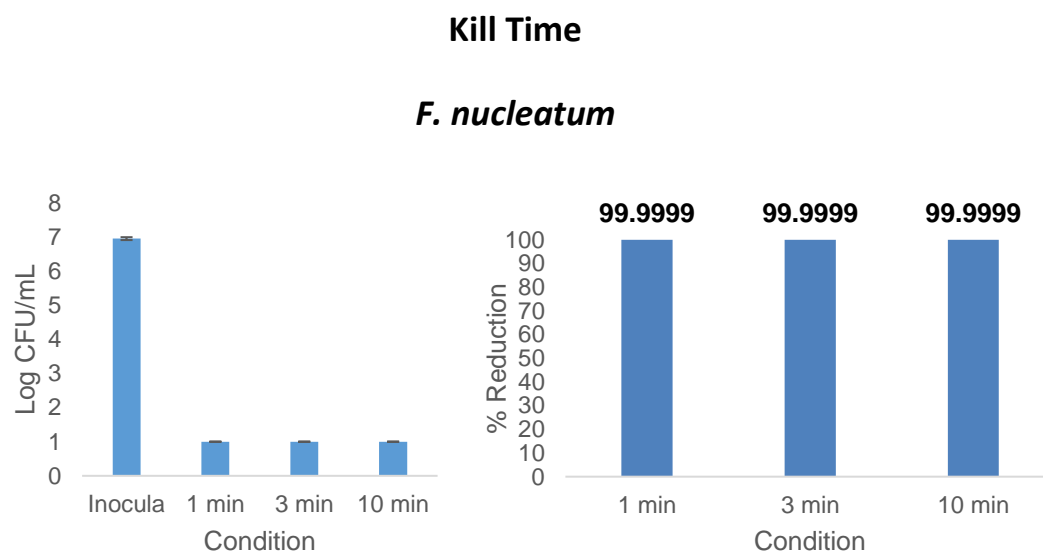


Figure 65 – Kill time results of Polident against *F. nucleatum*, the figure on the left shows the Log of CFU after the challenge and the right shows the percentage of reduction in the organism viability, the error bars show the standard deviation of three separate experiments.

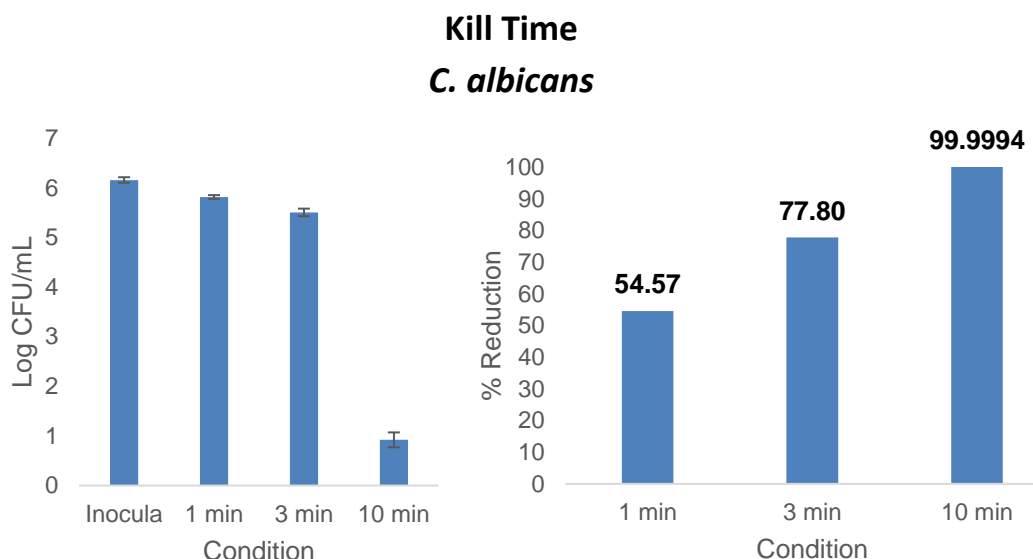


Figure 66 – Kill time results of Polident against *C. albicans*, the figure on the left shows the Log of CFU after the challenge and the right shows the percentage of reduction in the organism viability, the error bars show the standard deviation of three separate experiments.

Since Polident was effective in killing the chosen organisms this study evolved to a secondary phase where the Polident tablet and its ingredients will be studied alone and in different combinations to observe its antimicrobial properties and the role played by the ROS present.

When observing the MIC/MBC results for *C. albicans* the efficacy of the final product cannot be measured by the results of this experiment since the test concentration does not correspond to the concentration used in the household by the final consumer. The MIC/MBC experiment the test starting concentration was at 50% of the Polident tablet while for the kill time the starting concentration stood at 80%. The experiment setting does not permit us to change those concentrations unless a new tablet is prepared at higher concentration. For the experiments using the developed formulation these factors were taken into account.

In the kill time experiment, a significant difference between the placebo tablet and Polident tablet was not evident, a trend that was also observed in the MIC/MBC determination. The surfactant present in both formulations may have played a role in this finding and also the fact that

planktonic organisms were used and in this form they are much easier to eliminate than in the biofilm form. In order to ascertain if this is true, more experiments need to be done with the individual components of the Polident tablet in order to verify which ingredients are taking part on the microorganisms reduction.

3.3.3.2 MIC/MBC determination

The following figures summarized in the Table 17 show the absorbance at 590 nm of the organisms' suspensions after incubation for 24 hours in the presence of decreasing concentrations of the test agents. In the first instance the Polident and placebo tablets were tested, and this was followed by testing solutions of each of the main ingredients and subsequently test the same ingredients combined among them.

3.3.3.2.1 Polident and Placebo Tablets

On the first battery of tests it was shown that for the bacterium *S. mutans* (Figure 67), the minimum inhibitory concentration consisted of 12.50% of the total tablet solution contents and the same was observed for the placebo tablet. The anaerobic *F. nucleatum* (Figure 68) required a lower concentration of the tablet to inhibit its growth at 6.25% while for the placebo tablet MIC was 12.50% of the total tablet contents. Upon plating the MIC resulting suspensions none of it returned any colonies, meaning that the MBC has the same value as the MIC.

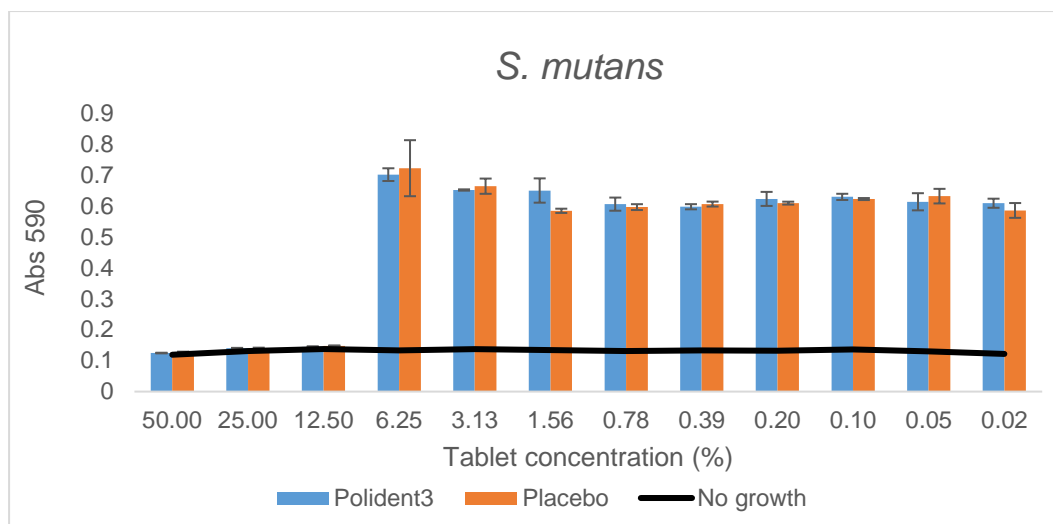


Figure 67 – Minimum inhibitory concentration absorbance at 590 nm results of Polident and placebo tablet solution against *S. mutans*. The black line represents no growth. If the absorbance is below this line means that the organism is not proliferating, the results show the average of three separate experiments done simultaneously and in triplicate using different inocula, the bars represent the standard deviation of three separate experiments.

For the yeast *C. albicans* (Figure 69) a higher concentration was needed to arrest the microorganism proliferation. The MIC determined was 50% of the tablet contents for both Polident and Placebo tablets and the subsequent MBC showed that the organism had not been eliminated at that concentration and Polident and placebo tablet had only a bacteriostatic effect.

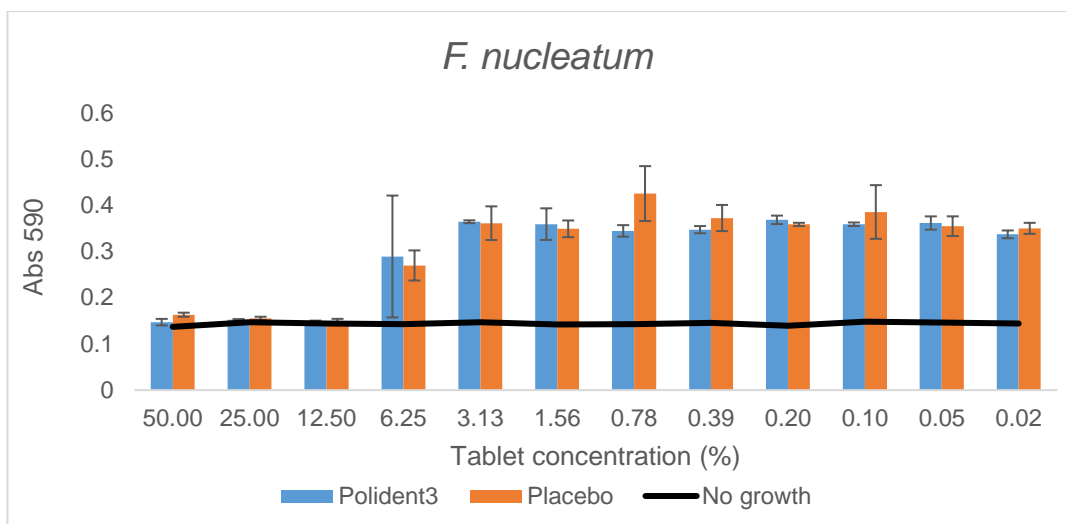


Figure 68 – Minimum inhibitory concentration of Polident and placebo tablet solution against *F. nucleatum*. The black line represents no growth. If the absorbance is below this line means that the organism is not proliferating, the results show the average of three separate experiments done simultaneously and in triplicate using different inocula, the bars represent the standard deviation of three separate experiments. At the concentration of 6.25 there is a enormous error bar due to pippeting error in one of the replicates after conclusion of the experiment the wells were empty.

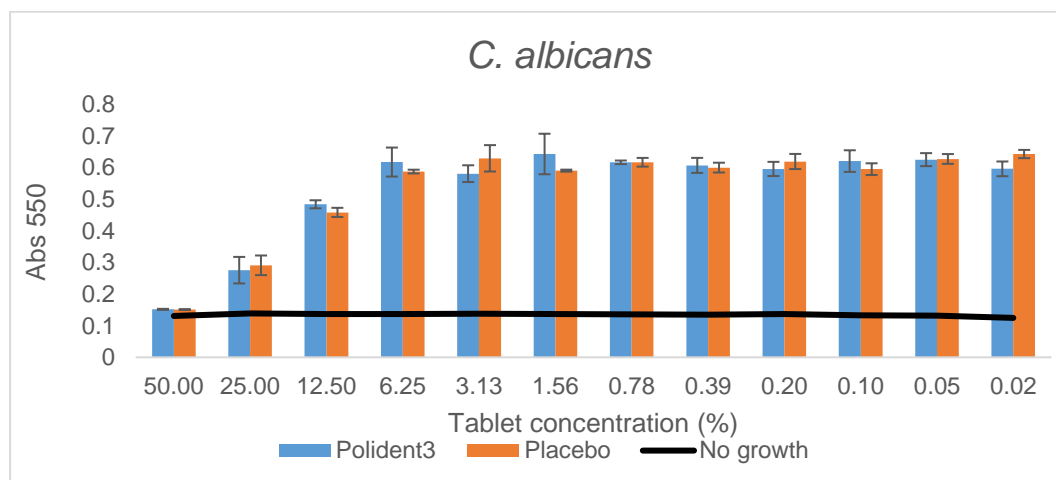


Figure 69 – Minimum inhibitory concentration of Polident and placebo tablet solution against *C. albicans*. The black line represents no growth. If the absorbance is below this line means that the organism is not proliferating, the results show the average of three separate experiments done simultaneously and in triplicate using different inocula, the bars represent the standard deviation of three separate experiments.

As expected, the Polident and placebo tablet solutions were effective in preventing growth of all the organisms tested and successfully eliminated *S. mutans* and *F. nucleatum*. This result shows that the tablet formulation on its own can eliminate the tested organisms, it is not a surprising result considering that the tablet contains MPS and surfactants that have antimicrobial properties not related to the generation of ROS. Also, the fact that bacteria in the planktonic form do not show the same strength than when living within a biofilm.

Table 17 – Minimum inhibitory concentration and Minimum Bactericidal Concentration Polident and placebo tablet solutions against the microorganisms tested. Against the fungus *C. albicans* none of the solutions at test concentration managed to kill the organism.

| Organism | MIC | MBC |
|----------------------------|------------|------------|
| <i>S. mutans</i> | 12.50% | 12.50% |
| <i>F. nucleatum</i> | 12.50% | 12.50% |
| <i>C. albicans</i> | 50% | N/A |

The results showed that Polident in the tested settings was antimicrobial towards *S. mutans* and *F. nucleatum* while for *C. albicans* was only fungistatic. This result shows that *C. albicans* is harder to eliminate than the other challenged organisms.

3.3.3.2.2 Investigation of Single Ingredient Solutions

Subsequently the solutions of the 6 main Polident ingredients were prepared to determine their MIC/MBC against *S. mutans* and *C. albicans*. The starting concentration of each solution was at the tablet concentration and the solutions were tested on their own and combined with the ingredients that showed antimicrobial potential on their own shown in the following tables.

Table 18 – Single ingredient solutions tested and respective concentrations and pH values.

| Tested Solutions | Test Concentration (g/ml) | pH |
|---------------------------------|---------------------------|-------|
| TAED | 5.99×10^{-4} | 6.35 |
| NaPC | 1.44×10^{-3} | 10.77 |
| CA | 3.60×10^{-3} | 1.64 |
| MPS | 2.16×10^{-3} | 1.93 |
| NaHCO ₃ | 6.88×10^{-3} | 8.89 |
| Na ₂ CO ₃ | 1.73×10^{-3} | 11.67 |

Table 19 – Combined ingredients solutions tested.

| Combined solutions | pH |
|--|-------|
| 5. TAED, NaPC, CA | 3.49 |
| 1. TAED, NaPC, CA, MPS, NaHCO ₃ , Na ₂ CO ₃ | 6.92 |
| 2. TAED, NaPC, CA, NaHCO ₃ , Na ₂ CO ₃ | 8.55 |
| 4. TAED, NaPC | 9.90 |
| 3. TAED, NaPC, NaHCO ₃ , Na ₂ CO ₃ | 11.12 |

The following Figures 70 – 75 present the MICs determine for each single ingredient against *S. mutans* and *C. albicans* in suspension. Each experiment was repeated three times in triplicates. The results represent the average of all results and the error bar represents the standard deviation.

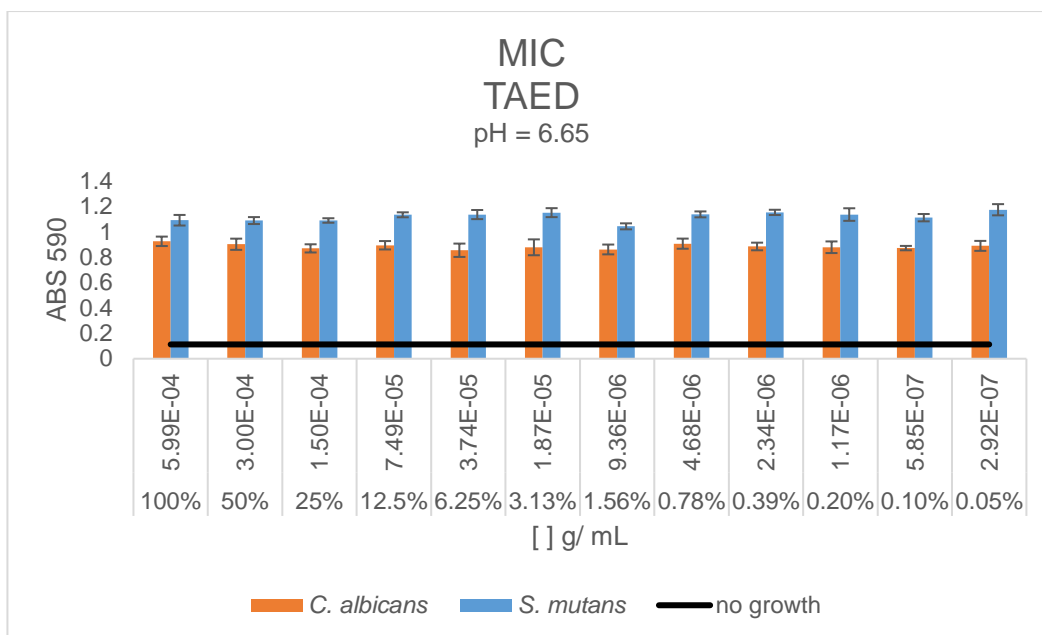


Figure 70 – Minimum inhibitory concentration results of TAED solution against *C. albicans* and *S. mutans*. The black line represents no growth. If the absorbance is below this line means that the organism is not proliferating, in this result since both organisms have proliferated means that an MIC or MBC could be detected at the tablet concentration for TAED. The results show the average of three separate experiments done simultaneously and in triplicate the bars represent the standard deviation.

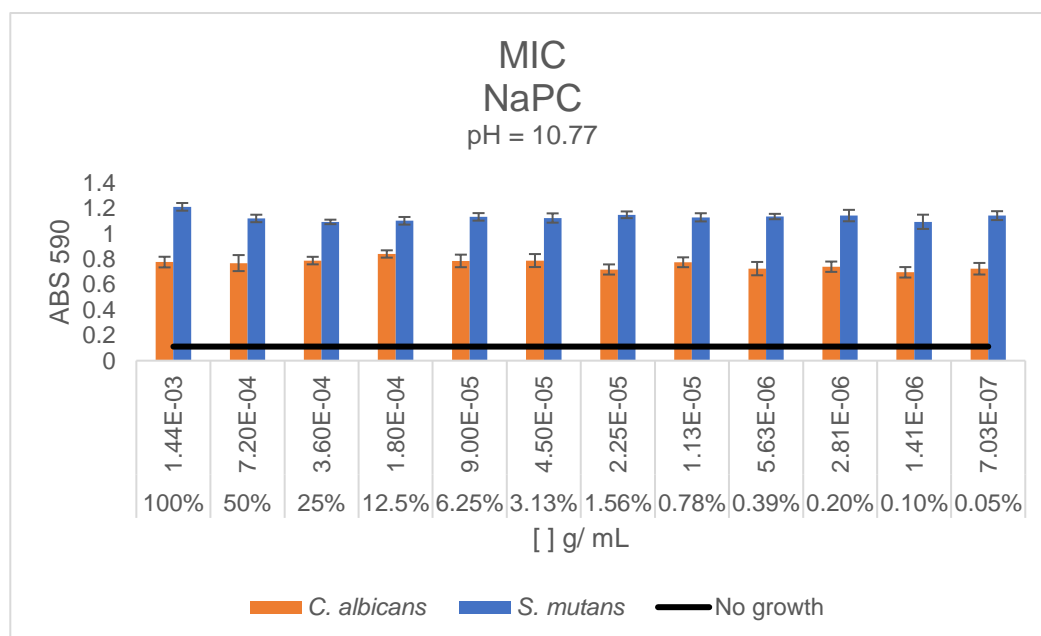


Figure 71 – Minimum inhibitory concentration of the NaPC solution against *C. albicans* and *S. mutans*. The black line represents no growth.

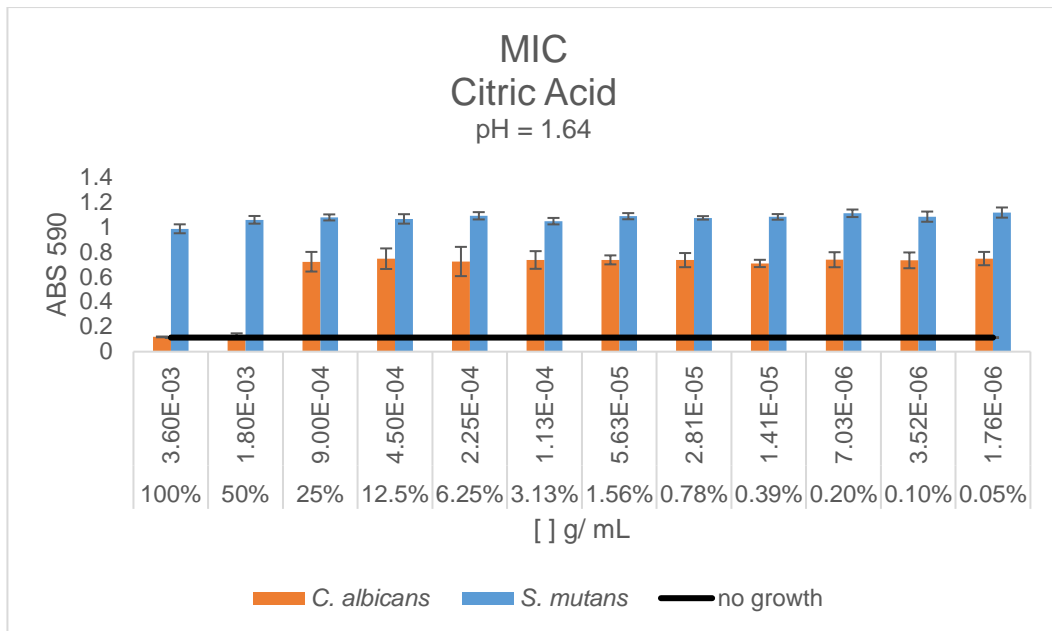


Figure 72 – Minimum inhibitory concentration of the citric acid solution against *C. albicans* and *S. mutans*. The black line represents no growth.

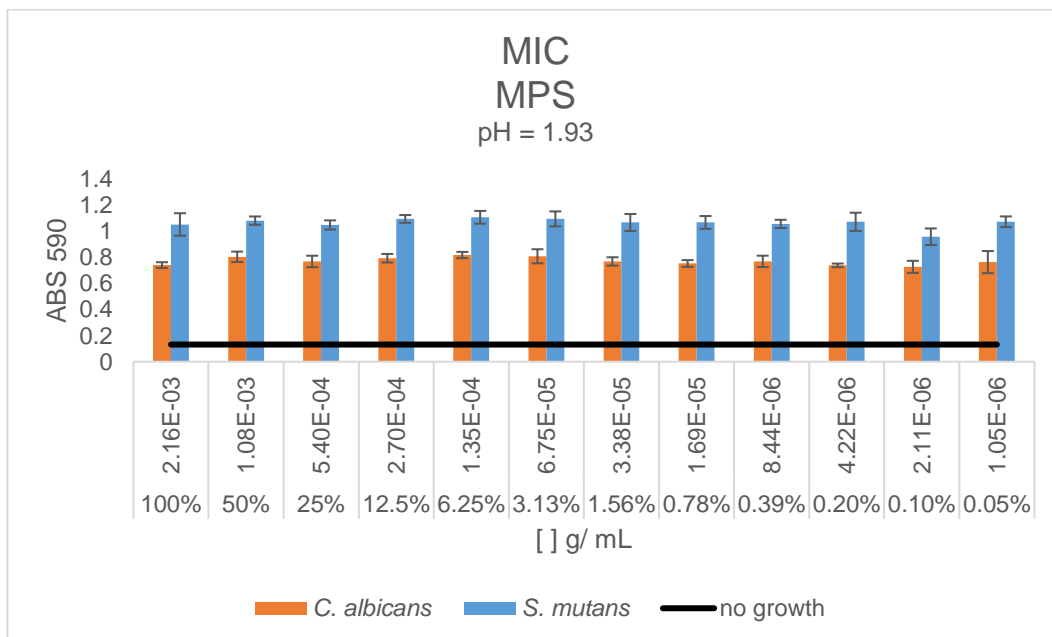


Figure 73 – Minimum inhibitory concentration of the potassium monopersulfate (MPS) solution against *C. albicans* and *S. mutans*. The black line represents no growth.

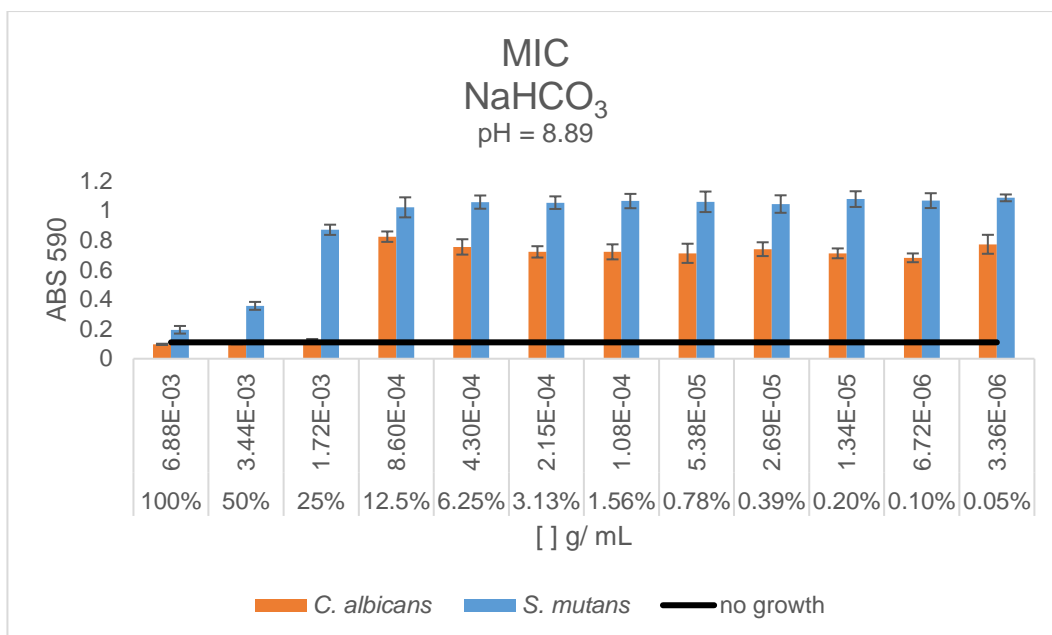


Figure 74 – Minimum inhibitory concentration of the sodium bicarbonate (NaHCO₃) solution against *C. albicans* and *S. mutans*. The black line represents no growth.

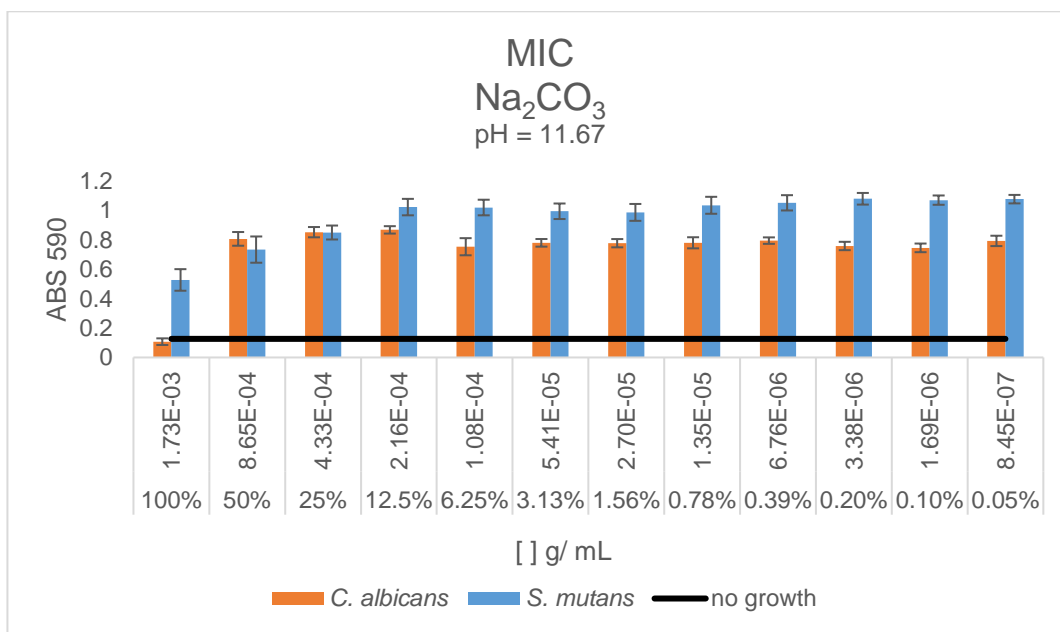


Figure 75 – Minimum inhibitory concentration of the sodium carbonate (Na₂CO₃) solution against *C. albicans* and *S. mutans*. The black line represents no growth.

When the single ingredients solutions were tested it was observed that for the bacterium *S. mutans* only one solution managed to successfully inhibit the growth of the organism at the tested concentration, the sodium bicarbonate solution (NaHCO₃) at a concentration of 6.88 x 10⁻³ g/mL (Figure

74). When an aliquot of the suspension was plated on agar it returned no colonies, meaning that at that concentration NaHCO_3 was bactericidal. Therefore, the MIC and MBC for NaHCO_3 was found to be 6.88×10^{-3} g/mL or 100% of the tablet content in NaHCO_3 .

For the yeast *C. albicans* citric acid, managed to inhibit its growth and proliferation (Figure 72). For NaHCO_3 the MIC was 1.72×10^{-3} g/mL and for sodium carbonate (Na_2CO_3) was 1.73×10^{-3} g/mL corresponding to 25% and 100% of the tablet contents for each ingredient respectively (Figures 74 and 75). Regarding citric acid the MIC was achieved with 50% of the tablet contents corresponding to 1.80×10^{-3} g/mL (Figure 72). Contrary to what occurred with *S. mutans*, when the suspensions were plated on agar all returned colonies, meaning that the solutions were only arresting the growth and proliferation of the organism and not killing it showing a bacteriostatic effect.

It is not surprising to see the single ingredients solution eliminating microorganisms on its own, this can be attributed to changes in the osmotic pressure and rapid changes in pH that can disrupt the normal functioning of microbial cells. Contrary to the formulated tablets these solutions were not buffered nor had its pH adjusted to the same levels as the tablet.

The results obtained with the Polident results were the MIC for *S. mutans* and *C. albicans* stood at 12.5% and 50% of the tablet concentrations respectively, the same fungistatic effect was observed towards *C. albicans* while *S. mutans* was eliminated at the MIC concentration agree with the results obtained by this experiment and lead us to the next stage were combinations of the single ingredients solutions were used to challenge the test organisms.

3.3.3.2.3 Investigation of combinations of Polident ingredients

When the combination of ingredients were tested, results shown in Figures 76 to 80 again *C. albicans* was more resistant than *S. mutans*. The solution containing all six ingredients (Figure 76) managed to successfully eliminate *S. mutans* at 50% of tablet concentration. Regarding *C. albicans* the solution arrest the growth of the organism at 100% concentration but did not eliminate it. From the ingredients in the solution only the carbonates (NaHCO_3 and Na_2CO_3) and citric acid showed bacteriostatic activity towards *C. albicans* while *S. mutans* was slightly affected by NaHCO_3 Na_2CO_3 showing reduced levels of proliferation.

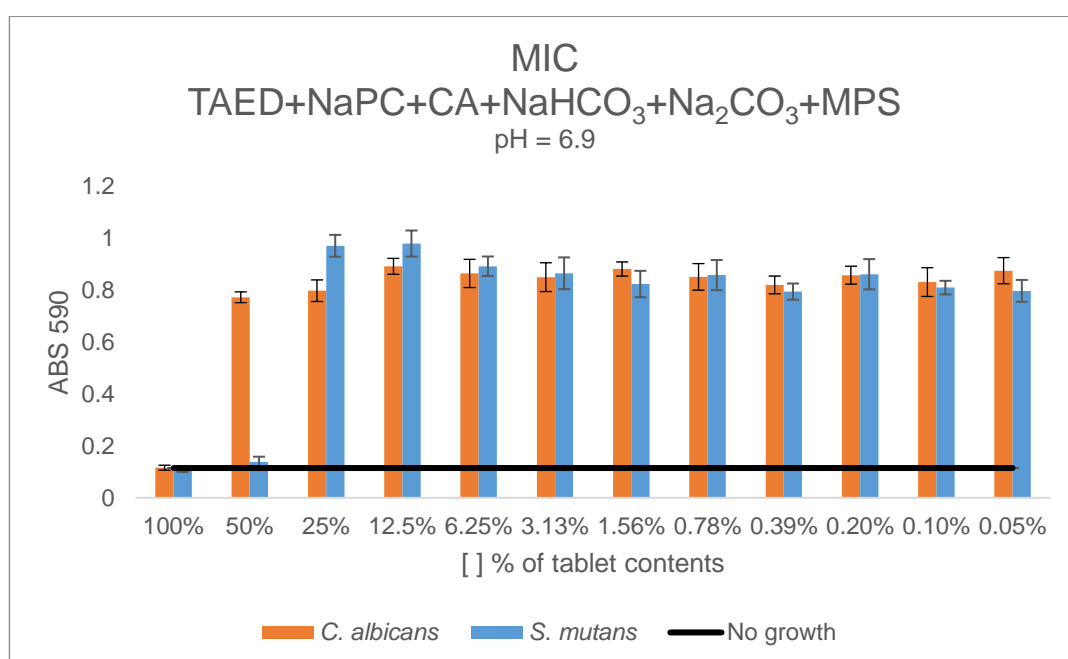


Figure 76 – Minimum inhibitory concentration of the combined solution of the TAED, NaPC, CA, NaHCO_3 , Na_2CO_3 and MPS combined solution for *C. albicans* and *S. mutans*. The black line represents no growth. The MIC for *C. albicans* was found at 100% concentration with no MBC at the tested concentration while for *S. mutans* MIC and MBC stood at 50%. The results show the average of three separate experiments done simultaneously and in triplicate the bars represent the standard deviation.

By removing MPS from the solution the pH increased from 6.9 to 8.6 and the antimicrobial activity towards *C. albicans* changed, the concentrations used in this experiment did not present an MIC value, but the proliferation of the organism was reduced as seen in the Figure 77, the

absorbance of the solution at 100% was 0.352 ± 0.02 , no growth control was 0.119 ± 0.001 and the growth control showed an average value of 0.844 ± 0.09 . This shows that at 100% concentration the organism was able to proliferate but not to the same extent as without challenge conditions.

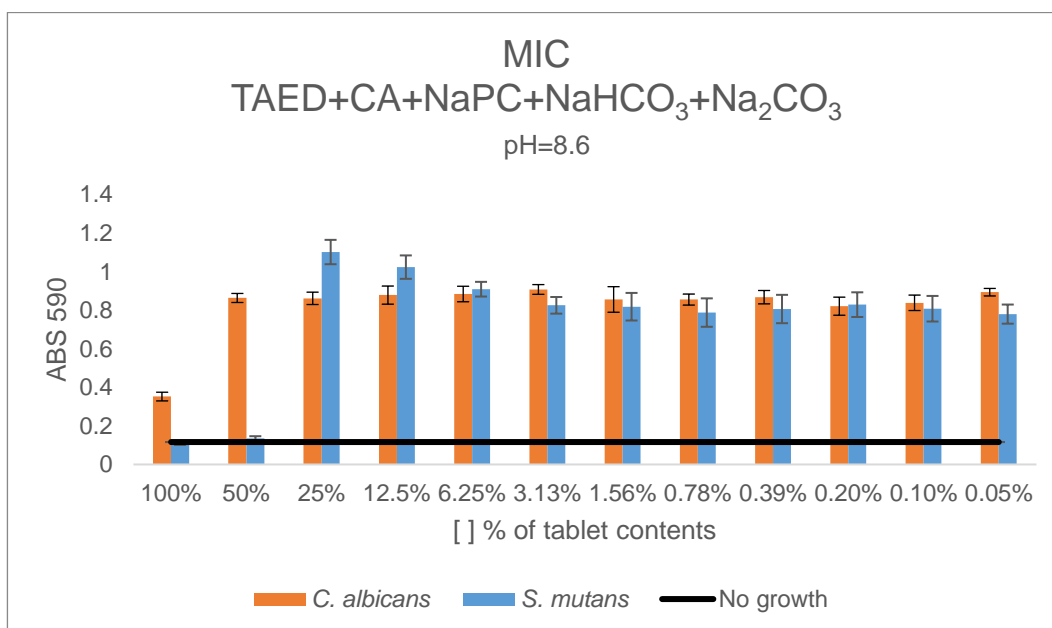


Figure 77 – Minimum inhibitory concentration of the combined solution of TAED, NaPC, CA, NaHCO₃ and Na₂CO₃ for *C. albicans* and *S. mutans*. The MIC and MBC for *S. mutans* were found at 50% of the tablet concentration while *C. albicans* had no MIC or MBC at the tested concentration. At 100% of the concentration there is reduced proliferation of *C. albicans* showing an absorbance of 0.3, uninhibited growth shows an absorbance around 0.85 and no growth roughly 0.1, this value between both shows that the organism was not killed but was not growing at the same levels show in the control experiment.

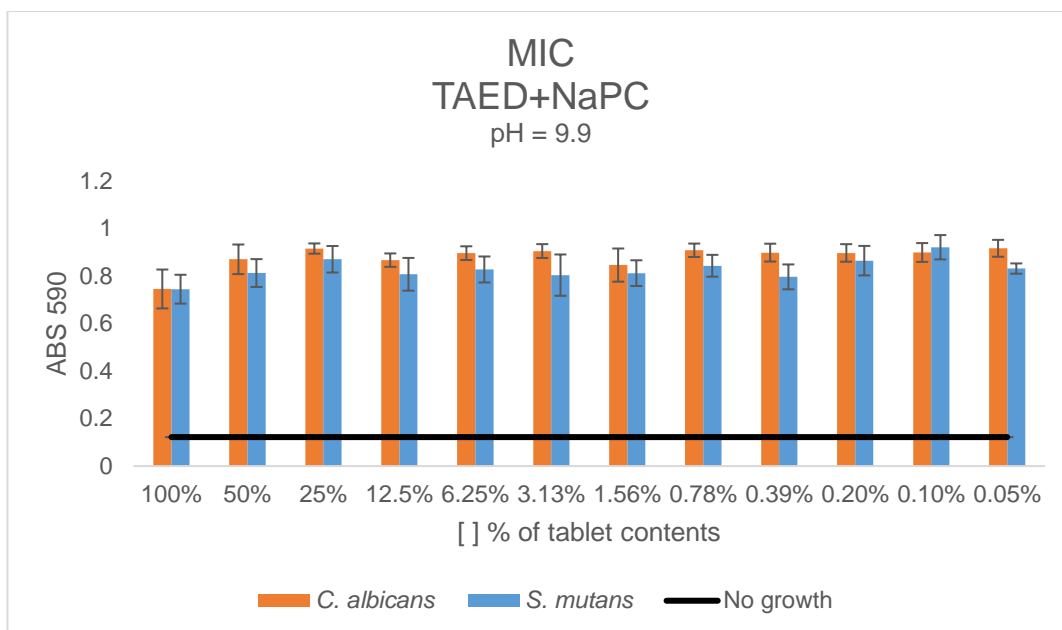


Figure 78 – Minimum inhibitory concentration of the combined solution of TAED and NaPC combined solution for *C. albicans* and *S. mutans*. The black line represents no growth.

The solution containing only TAED and NaPC (Figure 78) with pH of 9.9 was not antimicrobial towards the organisms used. It is significant to have present that the ideal pH for an optimal decomposition of peracetic acid that generates singlet oxygen is between 5.5 – 8.2^{79,80,85,119}. At a pH higher than 8.2 the hydrolysis of peracetic acid becomes the main route of decomposition leaving us with the final product of acetic acid and H₂O₂. So, there is no surprise that the combination of both actives, does not generate antimicrobial activity at the tested concentrations.

The solution containing the actives TAED and NaPC plus both carbonates (NaHCO₃ and Na₂CO₃) with a pH of 11.1 (Figure 79) show the existence of an MIC within the test concentrations for both of the tested organisms. As in previous results *C. albicans* was “stronger” with an MIC detected at 100% of the tablet concentrations and under these circumstances an MBC was not detected. While for *S. mutans* 50% of the solution contents were enough to eliminate it. By looking at the single ingredients solutions we can observe that both carbonates managed to inhibit the growth of *C. albicans* and NaHCO₃ and was effective in eliminating *S. mutans*. Although Na₂CO₃ affected the organism’s proliferation we cannot overlook that the

oxidation potential of H₂O₂ increases with pH showing that at a pH of 11 a much more reactive form of it is present.

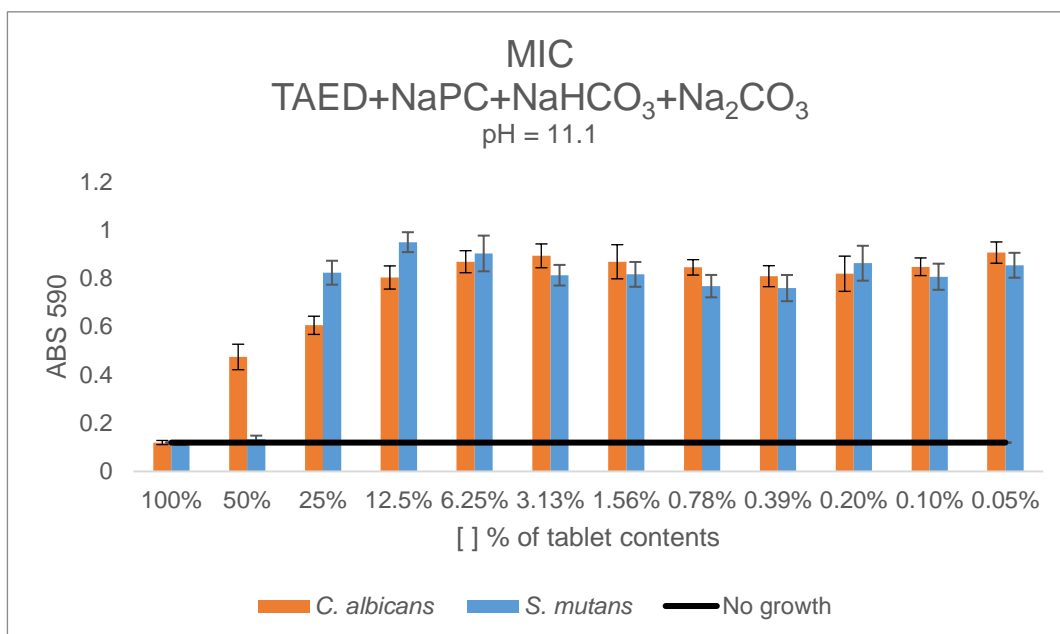


Figure 79 – Minimum inhibitory concentration of the combined solution of TAED, NaPC, NaHCO₃, Na₂CO₃ for *C. albicans* and *S. mutans*. The MIC for *C. albicans* was found at a concentration of 100% and no MBC was detected at the tested concentrations, while for *S. mutans* MIC and MBC was found at 50% of the tested concentration.

The combined solution of TAED, NaPC and citric acid (Figure 80) had the lowest pH of all the combined solutions at 3.5 and was only effective in eliminating *S. mutans*, at 100% of the tablet concentration. Interestingly, the single solutions of TAED, NaPC and citric acid did not present an MIC for this organism, at the tested concentrations showing that when combined they can make an antimicrobial solution. The citric acid solution, on its own, at 100% of the tablet concentration, managed to arrest the growth of *C. albicans* without eliminating it but in this case the combined solution showed no antimicrobial activity towards *C. albicans*.

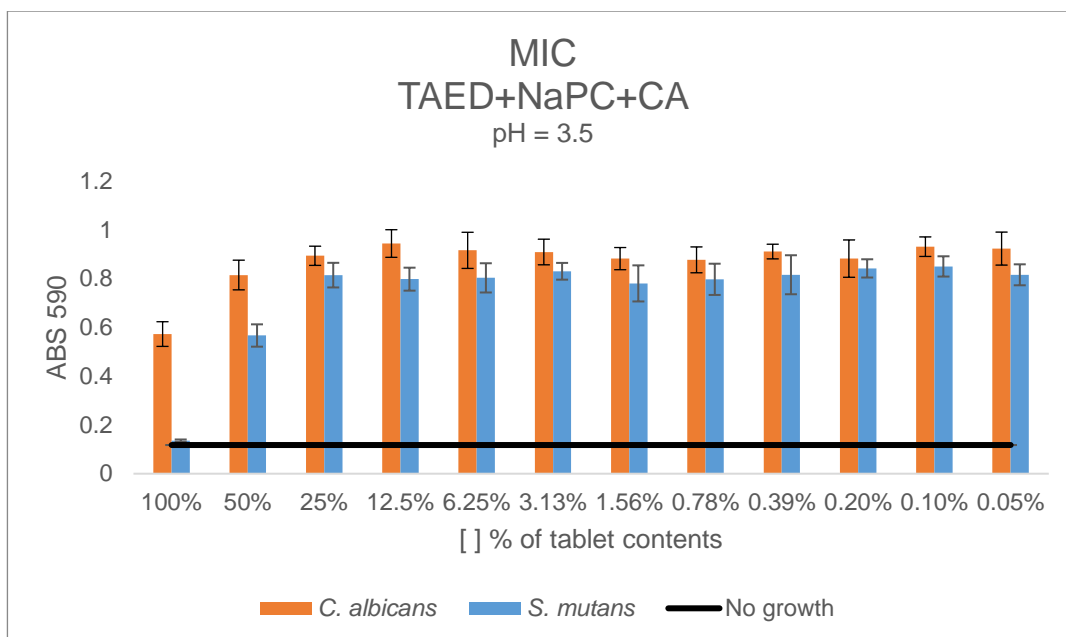


Figure 80 – Minimum inhibitory concentration of the TAED, NaPC and citric acid combined solution for *C. albicans* and *S. mutans*. No MIC nor MBC found for *C. albicans*, at the tested concentrations and 100% of tablet contents eliminated *S. mutans*.

By increasing the concentration of citric acid in the solution we effectively reduce the pH of the test solution. The solution of TAED, NaPC and Citric acid at the tablet concentration had a pH of 3.5 while the simplified formulations CA100 and CA 200 had pHs of 6.9 and 5.5 respectively.

Table 20 - pH and citric acid concentrations in the solutions containing TAED, NaPC and citric acid.

| Solution | [CA] g/ml | pH |
|------------------|-----------------------|-----|
| TAED + NaPC + CA | 2.16×10^{-3} | 3.5 |
| CA100 | 7.20×10^{-4} | 6.9 |
| CA200 | 1.44×10^{-3} | 5.5 |

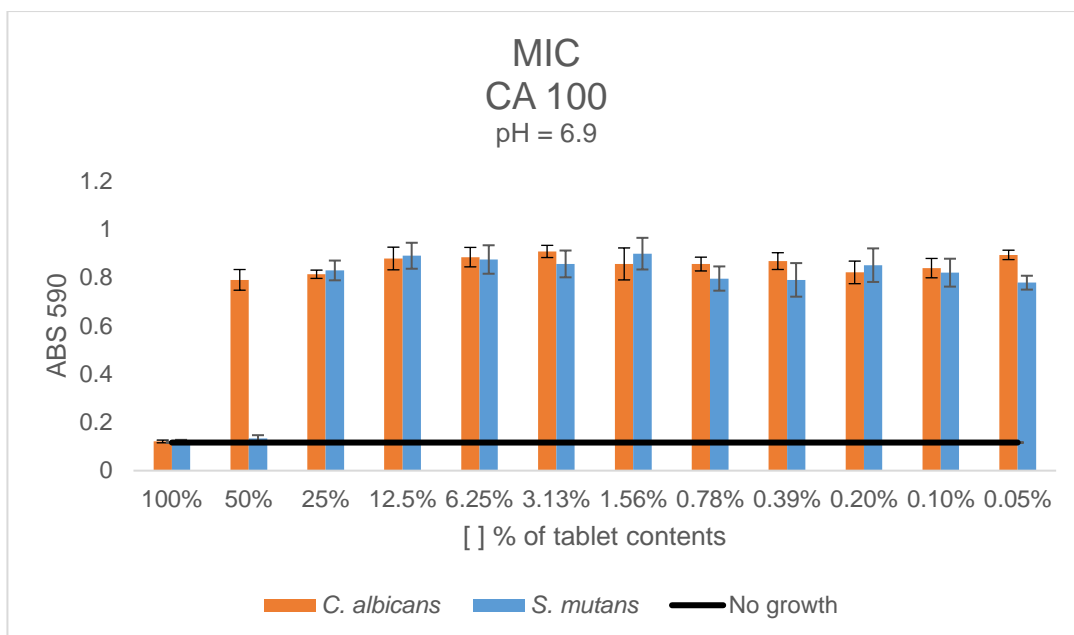


Figure 81 – Minimum inhibitory concentration of the formulation CA100 (pH=6.9), a combined solution of TAED, NaPC and citric acid, for *C. albicans* and *S. mutans*. The MIC for *C. albicans* was detected at 100% without MBC at the tested concentration while for *S. mutans* MIC and MBC were found at 50% of the tested concentration.

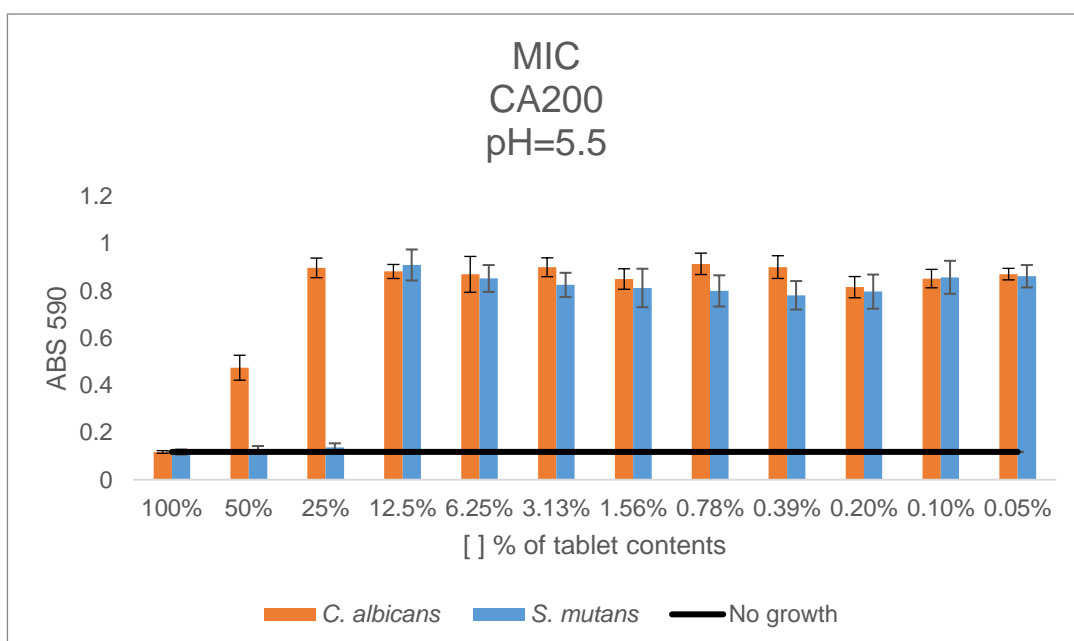


Figure 82 – Minimum inhibitory concentration of the formulation CA 200 (pH=5.5), a combined solution of TAED, NaPC and citric acid for *C. albicans* and *S. mutans*. The MIC for *C. albicans* was found at 100% without MBC while for *S. mutans* MIC and MBC was found at 50% of the tested concentration.

The two simplified formulations CA100 and CA200, shown in Figures 81 and 82 containing only TAED, NaPC and citric acid within the optimal pH

range for singlet oxygen generation managed to successfully eliminate *S. mutans*. CA 200 showed an MIC and MBC of 25% of the tablet contents (TAED and NaPC) and whereas CA100 had an MIC of 50% for *S. mutans*, for the yeast *C. albicans* only bacteriostatic effect was observed at 100% concentration for both solutions although CA200 at 50% affected the proliferation of *C. albicans*. By comparing with the TAED, NaPC, CA solution shown in Figures 80 - 82 we can observe that adjusting the pH to the optimal level 5.5 – 8.2 the antimicrobial activity of a solution containing TAED and NAPC towards *S. mutans* and to some extent to *C. albicans* was enhanced.

Table 21 - pH and citric acid concentrations in the solutions containing TAED, NaPC and citric acid. (NF – Not found)

| Solution | pH | MIC % | |
|------------------|-----|------------------|--------------------|
| | | <i>S. mutans</i> | <i>C. albicans</i> |
| TAED + NaPC | 9.9 | NF | NF |
| TAED + NaPC + CA | 3.5 | 100 | NF |
| CA100 | 6.9 | 50 | 100 |
| CA200 | 5.5 | 25 | 100 |

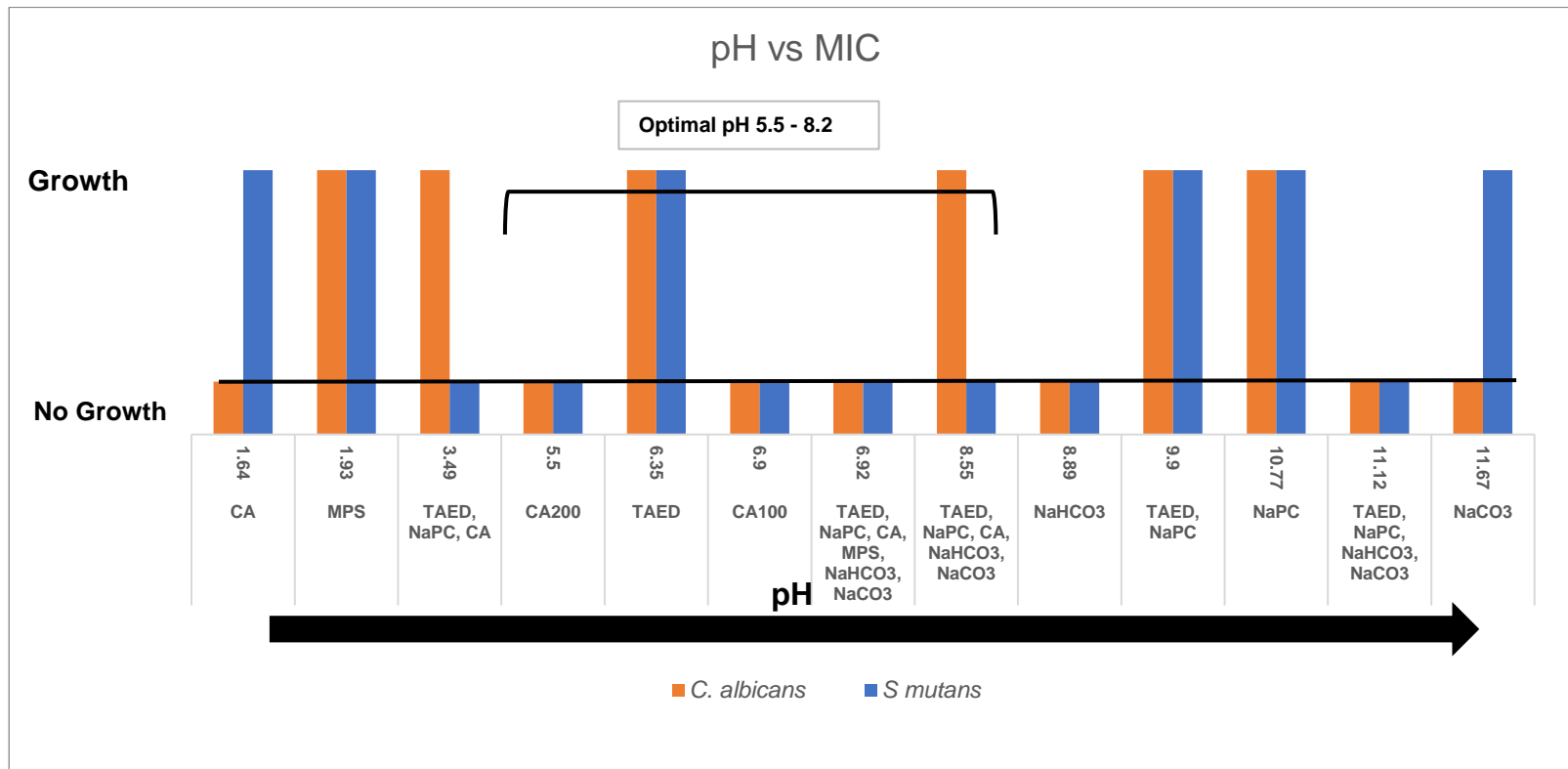


Figure 83 - Comparison between the pH of the tested solutions and the existence of MIC. The results are arbitrary. The bars represent growth or no growth (MIC) for *C. albicans* and *S. mutans* on the horizontal axis it is shown the solution and its pH with the pH increasing from left to right. The axis does not represent a linear pH scale.

In conclusion *S. mutans* was shown to be much easier to eliminate throughout the experiment. *C. albicans* was more resistant and harder to eliminate. This is assumed that this is because of its biology and capability to adapt to different environment conditions^{22,149}. It has been reported that *C. albicans* can effectively change the pH of its surrounding environment to promote its hyphal growth form increasing its survivability and virulence¹⁴⁹. *C. albicans* is a dimorphic fungus that can grow as a budding yeast and in hyphal form, its hyphal form is commonly associated with biofilm formation and pathogenicity¹⁵⁰.

The single ingredient solutions showed that *C. albicans* was only sensitive to citric acid, sodium bicarbonate and sodium carbonate, when using the combined solutions as expected the solutions containing those ingredients also presented antimicrobial activity. Whereas *S. mutans* was only eliminated by the sodium bicarbonate solution on its own. When combining the ingredients, the most effective solution from the original concentrations was the one containing all ingredients, that is also the one that best mimics the tablet. All ingredients together (TAED, NaPC, CA, MPS, NaHCO₃, Na₂CO₃) had an MIC and MBC of 50% for *S. mutans* and 100% to achieve an inhibitory effect towards *C. albicans*.

C. albicans was also more sensitive towards the solutions containing carbonates in a similar way as when challenged by the carbonates single ingredient solutions. *S. mutans* was eliminated by all tested solutions except the TAED and NaPC combined solution. The returned results also indicated some synergy in the antimicrobial effect by observing the results of the single ingredients solutions against the combined ingredients solutions (Figure 83).

By looking at MIC against the pH of the solutions shown in Figure 83 it is not evident that the pH of the tested solutions on its own possessed significant antimicrobial activity. The solutions containing TAED and NaPC, at the optimal pH, presented antimicrobial activity against both pathogens. To investigate in more detail the solution containing TAED, NaPC and citric acid had its pH adjusted by varying the citric acid concentration.

The solution of TAED and NaPC and the solution of TAED, NaPC and CA with pHs of 9.9 and 3.49 respectively did not present and MIC for *C. albicans* while CA 100 and CA 200 with pH of 6.9 and 5.5 respectively managed to successfully eliminate *S. mutans* and arrest the proliferation of *C. albicans*. This result shows that the generation of singlet oxygen plays an important role in the antimicrobial activity of the solutions. The solutions made of TAED, NaPC, NaHCO₃ and Na₂CO₃ also showed good antimicrobial properties to the same extent as the formulations generating singlet oxygen a fact that can be attributed to the high pH of 11.2 that makes H₂O₂ much more reactive.

The ¹H-NMR and fluorimetry studies proved that CA100 and CA200 generated singlet oxygen in the same fashion as the Polident tablet and the lower pH favoured the quantity and stability of singlet oxygen in solution.

3.3.3.3 Biofilm Studies

The initial biofilm studies performed using *C. albicans* to test the suitability of AlamarBlue for this assay proved successful as shown in Figure 84 and 85. AlamarBlue was capable of measuring the viability of the cells and the intensity of the signal increased with concentration of cells in the suspension.

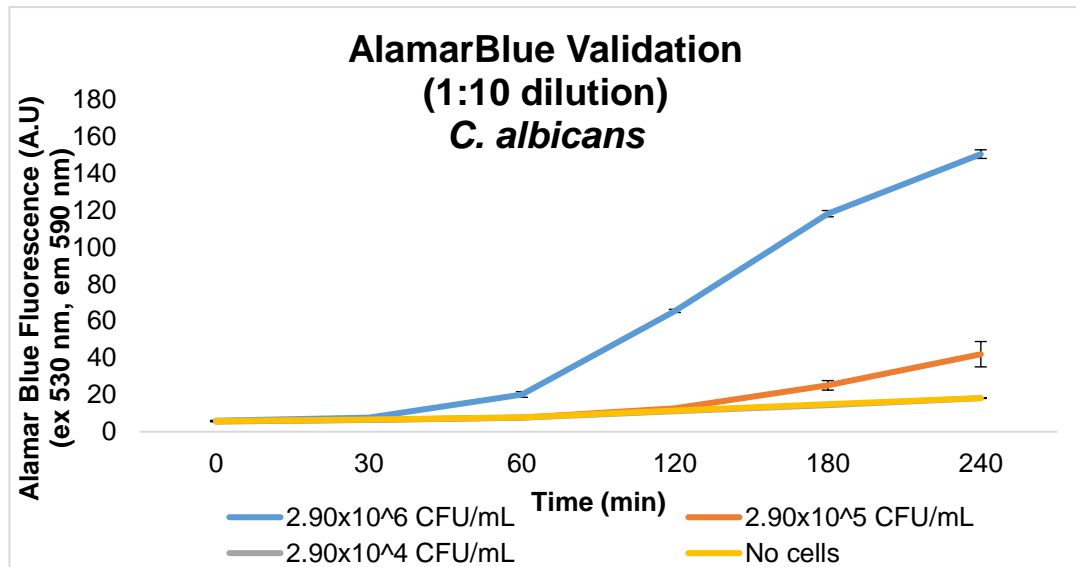


Figure 84 – AlamarBlue fluorescence using a suspension of *C. albicans* serially diluted 1:10. The yellow line represents the no growth control, in blue 2.90×10^6 CFU/ml suspension, in orange 2.90×10^5 and in grey (behind the yellow line) 2.90×10^4 CFU/mL. The error bar corresponds to the standard deviation of three separate experiments.

From the Figure 84 it can be observed that the intensity of AlamarBlue fluorescence increased with the number of cells in the suspension and the length of the incubation period.

The suspension containing 2.90×10^6 CFU/ml (blue line) started developing a signal after only 30 minutes while the suspension with 2.90×10^5 CFU/ml (orange line) at 120 minutes. The no growth control and the 2.90×10^4 CFU/ml suspensions both presented an identical signal throughout the experiment. This shows that at when inoculated at concentrations below 10^4 CFU/ml a 24 hour incubation period do not produce enough microbial cells to

originate a detectable fluorescence signal through reduction of AlamarBlue. After 180 minutes the two suspensions with the highest concentration had clear distinguishable fluorescent intensities 2.90×10^6 CFU/ml with 118.17 ± 1.69 A.U. and 2.90×10^5 CFU/ml with 25.15 ± 2.58 A.U. The highest fluorescence was achieved in our final measurement but the trend on the graph suggest that it would continue to increase.

To assess the AlamarBlue fluorescence a new experiment was set but, in this instance, using 1:2 dilutions of the inoculum, the results are shown in the Figure 85. In the first test using the MBEC™ and PMMA disks, showed that a robust biofilm could be obtained.

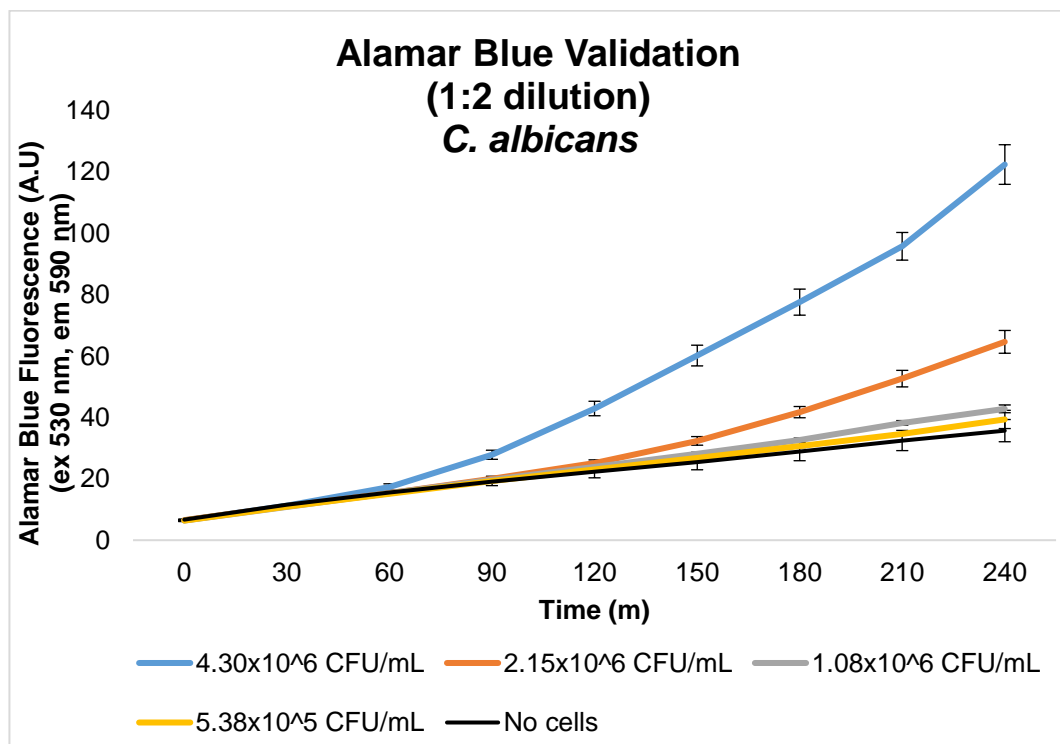


Figure 85 – AlamarBlue fluorescence using a suspension of *C. albicans* serially diluted 1:2. The black line represents the no growth control, in blue 4.30×10^6 CFU/ml suspension, in orange 2.15×10^6 , in grey 1.08×10^6 CFU/mL, in yellow 5.38×10^5 CFU/mL. The error bar corresponds to the standard deviation of three separate experiments.

By comparing figures 84 and 85 it was observed that by increasing the number of cells present in the suspension the fluorescence signal also increased. The lowest concentration in this test was 5.38×10^5 CFU/mL which had a signal higher than our negative control. The intensity of the fluorescence increased throughout the experiment with time.

Based on the results of this experiment it was decided to prepare inoculum suspensions of 10^6 - 10^7 CFU/mL in future experiments and use a 4 hour incubation period to measure biofilm viability with AlamarBlue.

In the next phase of experiments, it was assessed the suitability of the MBEC™ device (uncoated and HA coated) and the Lucitone PMMA disks to grow the biofilms.

In a similar fashion as for the previous experiment, the inoculum was prepared and serially diluted (1:10) in a 96 well plate and 24 well plate (note: 96 well plate to be used with the MBEC device and 24 well plate with the PMMA disks). Once the inoculum was prepared the lids or disks were placed in the suspension and incubated for a 24 hour period (37 °C, 120 RPM) This time was used to replicate customer use and normal denture cleaning. Followed by a 4 hour incubation with 10% AlamarBlue in BHI.

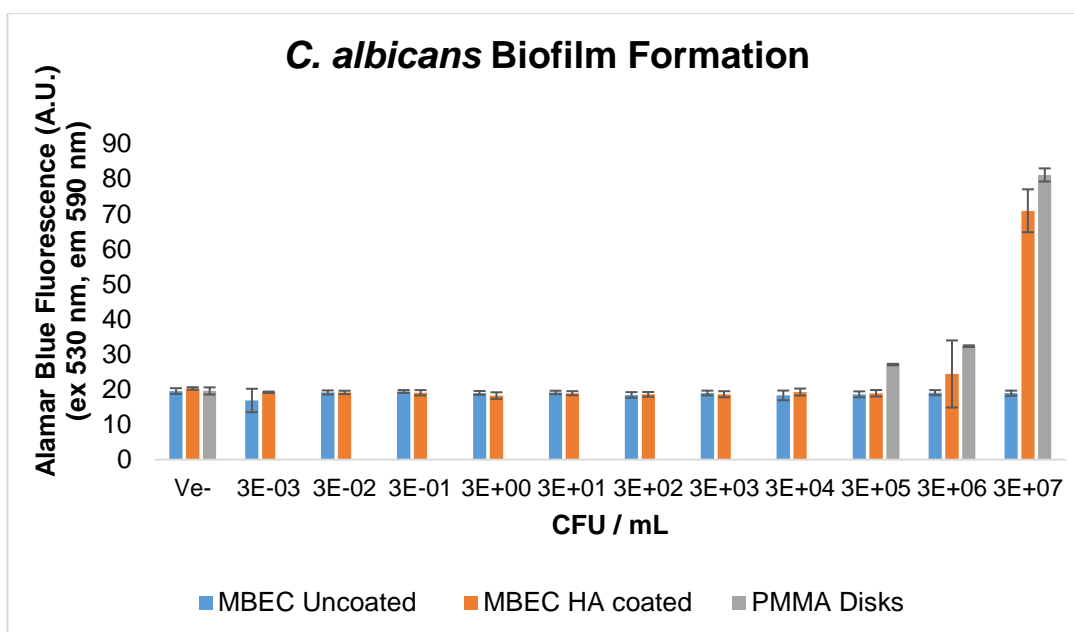


Figure 86 - AlamarBlue fluorescence (ex 530, em 590) after a 4 hour incubation with the *C. albicans* 24 hours biofilm growth using different *C. albicans* concentrations in different substrate. In blue MBEC uncoated (polysterene), in orange hydroxyapatite (HA) coated MBEC and in grey PMMA disks.

The Figure 86 shows the viability of the resulting biofilm on each substrate used. By comparing the returned results against the no growth control, it was concluded that the uncoated MBEC device was not suitable for this experiment since no biofilm formation was detected at the tested inoculum concentrations. The HA coated MBEC device and the PMMA disks proved to be suitable substrates for biofilm growth if an inoculum of at least 10^{-6} to 10^{-7} CFU/mL was used. Ideally to replicate the denture biofilm a material like PMMA should be used but the MBEC devices that are commercially available at the time of the experiment did not have a PMMA coated version. HA was the chosen substrate because it closely mimics the tooth material allowing us to give an ideal environment for the adhesion and growth of dental plaque.

The PMMA disks (grey) started to show a fluorescent signal 27.12 ± 0.21 against the 19.59 ± 1.01 of the no growth control, with inoculum concentrations of 3.0×10^5 CFU/mL. At the concentration of 3.0×10^6 cells/mL

a biofilm also started to develop on the HA coated MBEC device showing a fluorescence of 24.44 ± 9.56 against 20.31 ± 0.35 of the no growth.

At the highest concentration of 3.0×10^7 cells/mL the fluorescent signal greatly increased for HA coated MBEC device and PMMA disks at 70.97 ± 6.11 A.U. and 81.18 ± 1.87 A.U. respectively.

This experiment enabled to further narrow the necessary conditions for the subsequent experiments by showing that the HA coated MBEC device and the PMMA disks were suitable for biofilm growth and further detection of its viability using AlamarBlue. AlamarBlue required a concentration of cells above 10^4 CFU/ml to produce a detectable fluorescent signal. The MBEC device and the PMMA disks require a concentration of 10^7 CFU/mL to grow the desired biofilm in 24 hours. Not surprisingly the human saliva contains a total count of microorganism between 10^8 to 10^9 CFU/ml, the CFU of each specific bug will vary according to different factors such as diet, demographics, age^{151,152}.

Then using the HA coated MBEC device the biofilm removal properties of the Polident, Placebo and CA200 were studied using AlamarBlue, to measure biofilm viability of single species biofilms of *C. albicans* and *S. mutans*, and the PMMA disk were used to observe its effects on the removal of a dual species biofilm using Scanning Laser Confocal Microscopy and SEM, as mentioned in the methods section.

Of the 96 pegs available in the MBEC device 88 device pegs were used to grow identical biofilms and then challenged by immersing the biofilm for 10 minutes in the challenge solutions at room temperature. The unused pegs represent the negative control (no growth) and were used as reference no biofilm or cells present. These controls showed an average fluorescence throughout the experiments of 30.80 ± 4.38 A.U. for *S. mutans* and 35.46 ± 4.05 A.U. for *C. albicans*. Furthermore sixteen pegs, with biofilm, were not challenged with the test solutions to represent the maximum growth of

the biofilm in 24 hours at 37 °C and presented a fluorescence of 164.60 ± 8.85 A.U. for *S. mutans* biofilm and 178.60 ± 11.45 A.U.. Then using the no growth and maximum growth as reference the data was normalized and the results presented as surviving/removed biofilm shown in Figures 88 and 90 for *S. mutans* and *C. albicans* respectively.

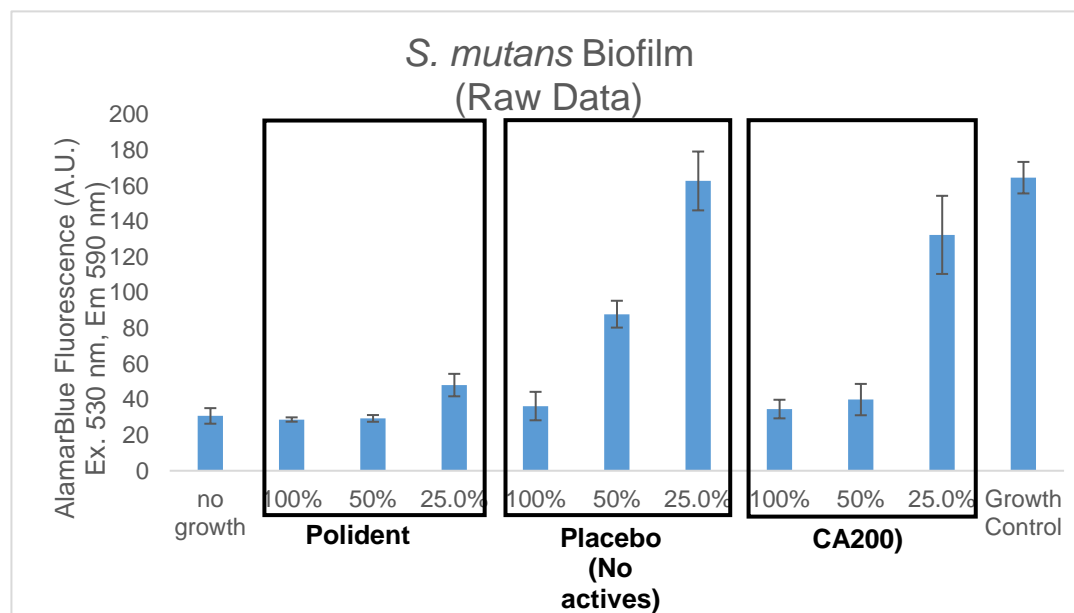


Figure 87 - AlamarBlue fluorescence of a 24 hours biofilm of *S. mutans* biofilm grown on HA coated MBEC device after 10 minute challenge by Polident, Placebo tablet and CA200 formulation. No growth represents the negative control with no cells and the growth control an unchallenged biofilm. The data corresponds to three separate experiments with 8 replicates per condition with the standard deviation.

Figure 87 shows the AlamarBlue fluorescence of a 24-hour biofilm of *S. mutans* grown using the MBEC device. After normalizing the data (figure 88) using growth control as 100% growth and no growth 0% reference. We can observe that Polident was very effective in removing biofilm, a concentration between 25 - 50 % of the total tablet contents was sufficient to remove all of the biofilm in the pegs. The lowest tested concentration 25% removed part of the biofilm but 13% of biofilm remained attached to the pegs. The placebo tablet also removed some biofilm at 100% of the total tablet concentration with only 4% of the biofilm remaining attached to the pegs, by decreasing the concentration to 50% and 25% the amount of remaining

biofilm increased to 43% and 99% respectively. The formulation CA200 at 100% concentration virtually removed all of the *S. mutans* biofilm leaving only 2.88% attached to the pegs while the placebo formulation at 100% concentration removed all but 4.14% of the biofilm. When comparing these two formulations CA200 appeared to be more effective in biofilm removal since at 50% concentration left 6.86% biofilm in the pegs and placebo left 42.7%, 25% of the tablet contents also presented the same trend for both of the test solutions. The organism *C. albicans* in the biofilm form also appeared to be more resistant than *S. mutans* as observed in the experiments with planktonic organisms (Figure 89). In this instance the placebo tablet only managed to remove 53% of the biofilm and 25% and 12.5% only removed 6% and 9% of biofilm respectively.

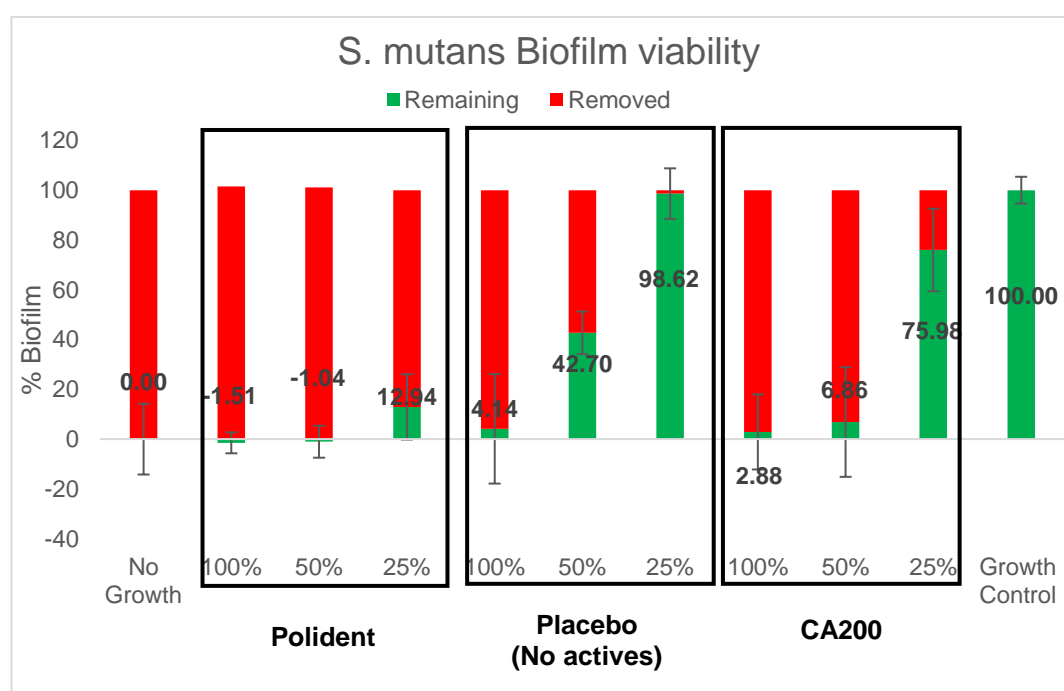


Figure 88 - Percentage of reduction of a 24 hours *S. mutans* biofilm reduction after 10 minute challenge by Polident, Placebo tablet and CA200 formulation. Red shows the removed biofilm and green the viable biofilm that reacted with AlamarBlue. The numbers inside the columns correspond to the percentage of remaining biofilm. The data corresponds to three separate experiments with 8 replicates per condition plotted with the relative error.

The formulation CA200 at 100% concentration was effective in removing all of the biofilm and at 50% removed 64.8% of biofilm and 25% removed 11.5% (Figure 89). Polident showed a similar efficacy as against the *S. mutans* biofilm where 25% of the tablet contents were enough to completely remove the biofilm, but in this instance the placebo tablet left 47% of the biofilm attached to the pegs at 100% of tablet contents. Polident tablet as with *S. mutans* 25 - 50% of the tablet contents successfully removed all of the biofilm and the CA200 formulation removed all of the biofilm at 100% concentration.

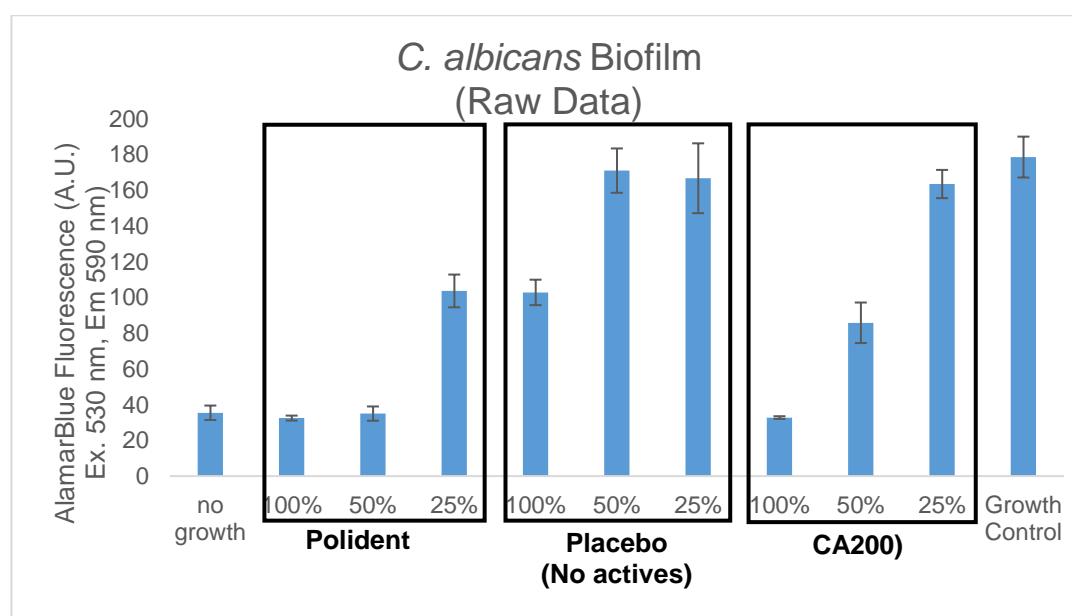


Figure 89 - AlamarBlue fluorescence of a 24 hours biofilm of *C. albicans* after 10 minute challenge by Polident, Placebo tablet and CA200 formulation. No growth represents the negative control with no cells and the growth control an unchallenged biofilm. The data corresponds to three separate experiments with 8 replicates per condition plotted with the standard deviation.

The results obtained from the biofilm experiment completely agree with the MIC and MBC previously determined for the tested solutions. The placebo tablet was more effective against planktonic organisms than in the biofilm form. It had an MIC and MBC for *S. mutans* at 12.50% and MIC of 50% for *C. albicans* but that did not correlate with biofilm removal. The Polident tablet also presented the same MIC and MBC for *S. mutans* at 12.50% of the contents and 50% for *C. albicans* MIC. But regarding biofilm

removal 50% of the total contents were sufficient to completely remove the biofilm. Since the main difference between both formulations relies on the generation of ROS it must be assumed that the generation of ROS plays a crucial role in the mechanism for the removal of biofilms.

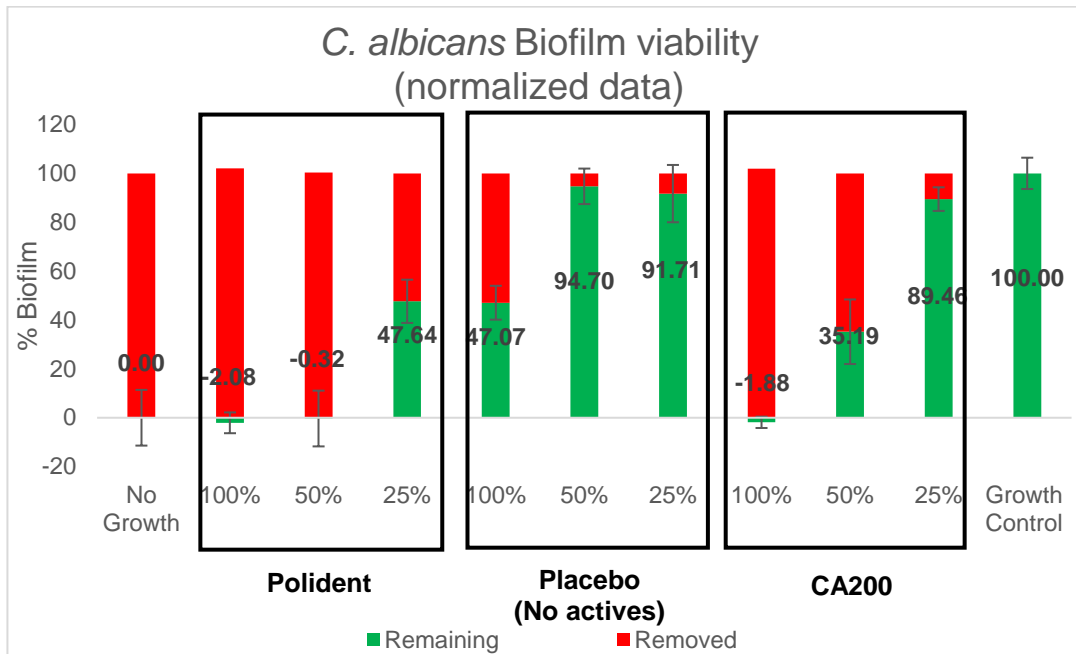


Figure 90 - Percentage of reduction of a 24 hours *C. albicans* biofilm reduction after 10 minute challenge by Polident, Placebo tablet and CA200 formulation. Red shows the removed biofilm and green the viable biofilm that reacted with AlamarBlue. The numbers inside the columns correspond to the percentage of remaining biofilm. The data corresponds to three separate experiments with 8 replicates per condition plotted with the relative error.

The CA200 solution capable of generating ROS also managed to remove both biofilms at 100% concentration proving that on its own ROS species possess the capability of removing biofilms of *C. albicans* and *S. mutans*.

3.3.3.4 Biofilm Visualization

The live and dead staining using CLSM (Figures 91 – 94) and SEM images (Figures 95 – 101) further clarified the results obtained. The placebo tablet left a considerable amount of biofilm on the PMMA disk after challenge and there was a mixture of live (green) and dead (red) cells. While Polident and CA200 managed to remove most of the biofilm and the remaining cells were mainly dead or with compromised membranes.

The MIC/MBC determination experiments a significant difference between the placebo and Polident tablet solutions was not evident while the biofilm studies were essential to scrutinize differences between the antimicrobial activity of both solutions. This shows that there is a significant difference in biofilm removal by the tested solutions. The tablet solution, dissolved as per manufacturer instructions, successfully removed *C. albicans* biofilm below detectable levels while the placebo solution only eliminated around 43% of biofilm.

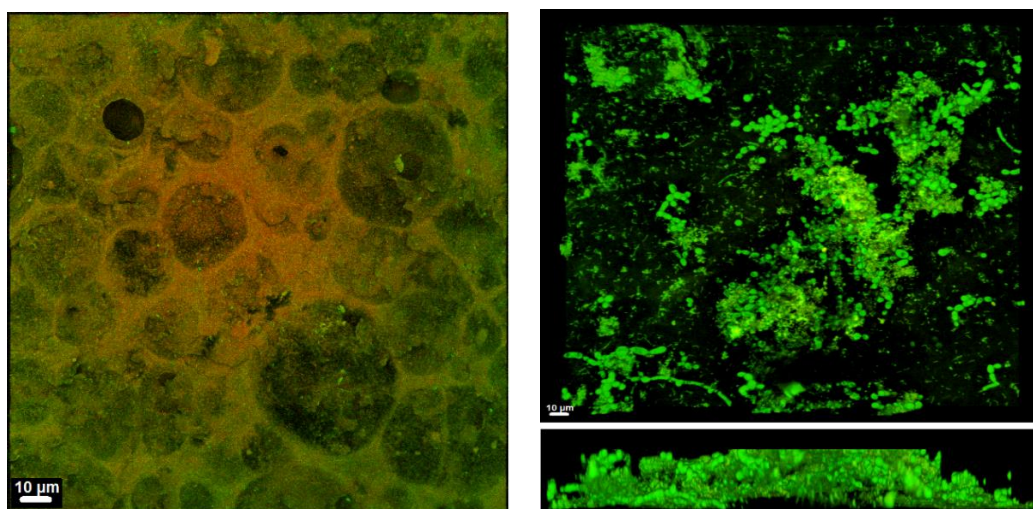


Figure 91 – CLSM image left PMMA disk without biofilm. Right PMMA disk with unchallenged biofilm of *C. albicans* and *S. mutans*. The bottom image shows a side view of the biofilm at the same scale. The green cells are live cells. The scale bar represents 10 μm.

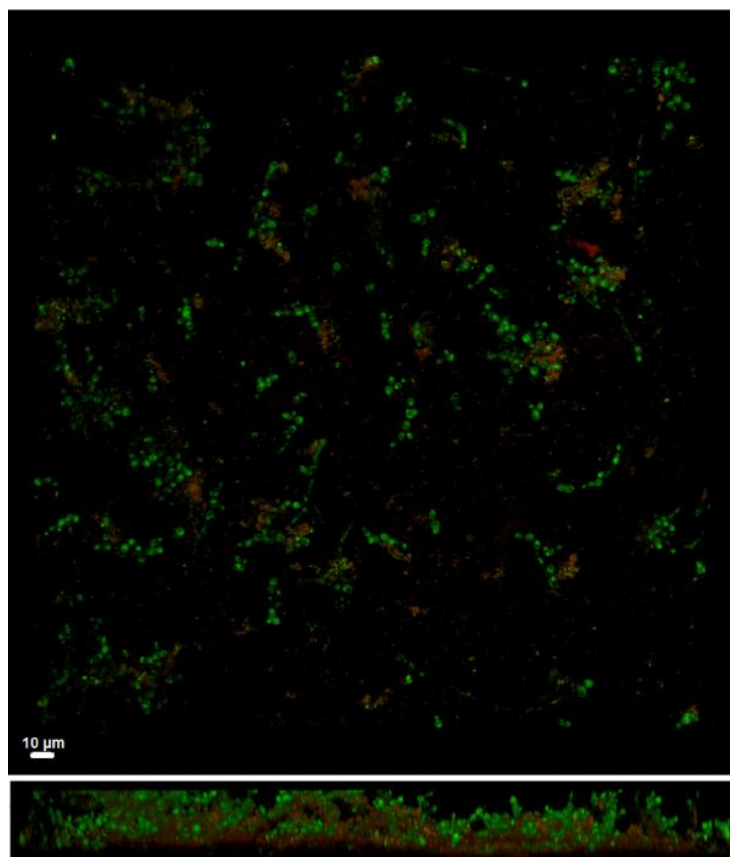


Figure 92 – CLSM image of *S. mutans* and *C. albicans* biofilm in a PMMA disk after 10-minute challenge with the Placebo tablet. The green are the live cells and dead / compromised membrane cells in red. The bottom image shows a side view of the same biofilm. The scale bar represents 10 μm .

By comparing the image after challenge by the placebo tablet solution (Figure 92) with the unchallenged biofilm (Figure 91 right) it was observed that some of the biofilm was removed and the remaining biofilm is a mix of live and dead cells and the majority of remaining cells seem to be of *C. albicans*.

In contrast to the Polident tablet and the CA200 formulation removed more biofilm and the majority of remaining cells are dead (red) (Figures 93 and 94 respectively).

The placebo tablet containing the surfactant and other ingredients is capable to remove biofilm but not of killing the cells within the biofilm nor completely dislodge it. The Polident tablet with the capability of generating ROS was very effective in the removal and killing of the biofilm, the Figure 93

shows that the majority of remaining cells were dead and the side view of the biofilm also confirms that the stack of cells is considerably smaller than the ones for the growth control and placebo tablet.

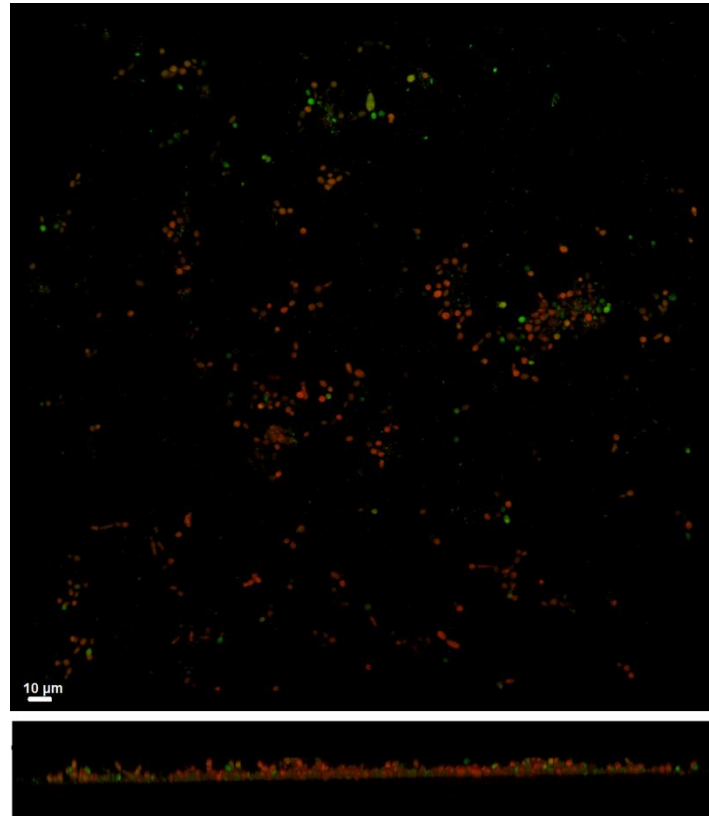


Figure 93 – CLSM image of *S. mutans* and *C. albicans* biofilm in a PMMA disk after 10 minute challenge with the Polident tablet. Bottom side view of the same biofilm. The green are the live cells and dead / compromised membrane cells in red. The scale bar represents 10 μm.

The formulation CA200 that solely relied in the generation of singlet oxygen to remove biofilm killed the cells and left a biofilm with dead cells shown in Figure 94.

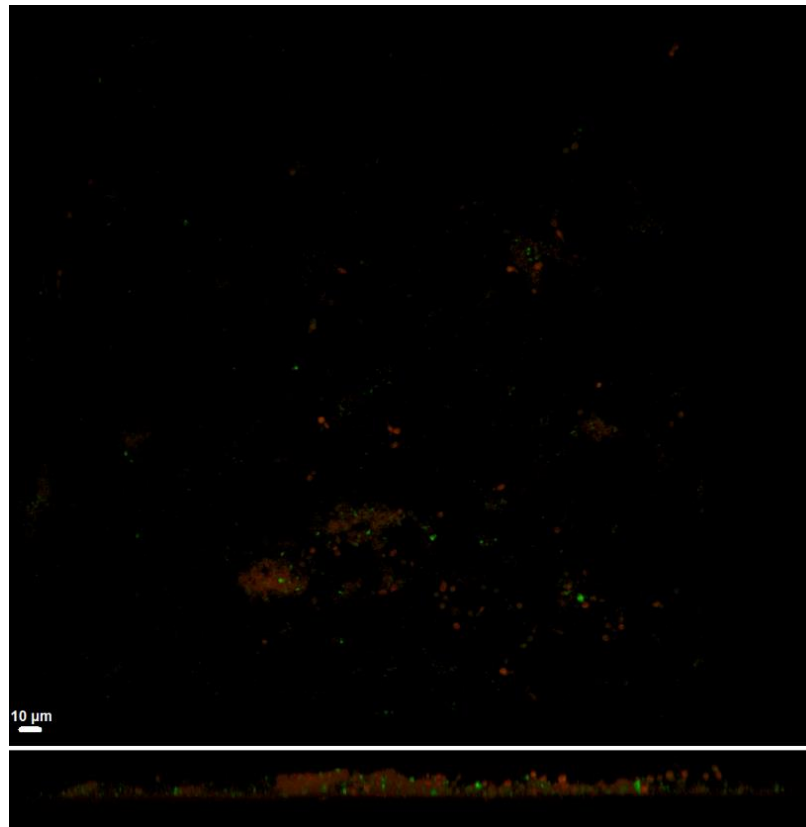


Figure 94 – CLSM image of *S. mutans* and *C. albicans* biofilm in a PMMA disk after 10 minute challenge with CA200 formulation at 60x magnification. The image in the bottom is a side view of the same biofilm. The green are the live cells and dead / compromised membrane cells in red. The scale bar represents 10 μm.

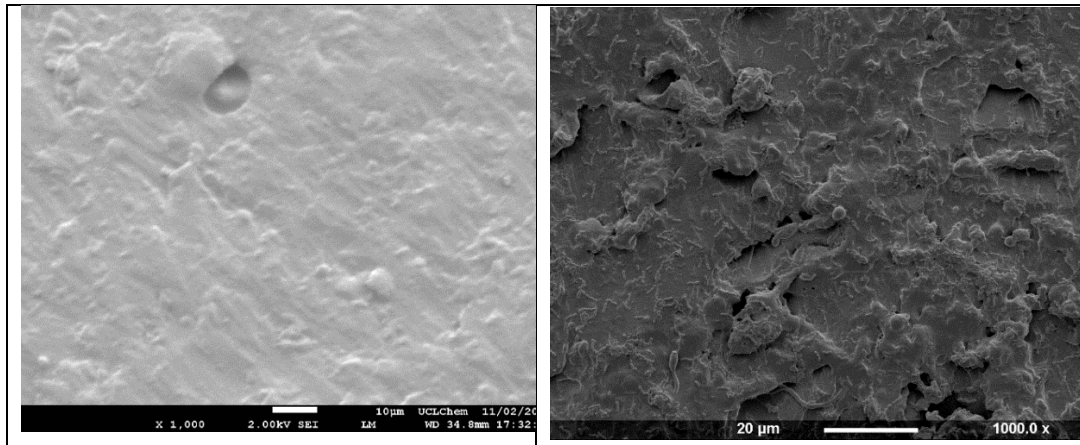


Figure 95 - 1000 x SEM images of PMMA disks. Left image clean PMMA disk, right PMMA disk with dual species biofilm of *C. albicans* and *S. mutans*. The organism *C. albicans* is easy to identify due to its big size, *S. mutans* is much smaller and the cells grow in chains. See Figure 92 below.

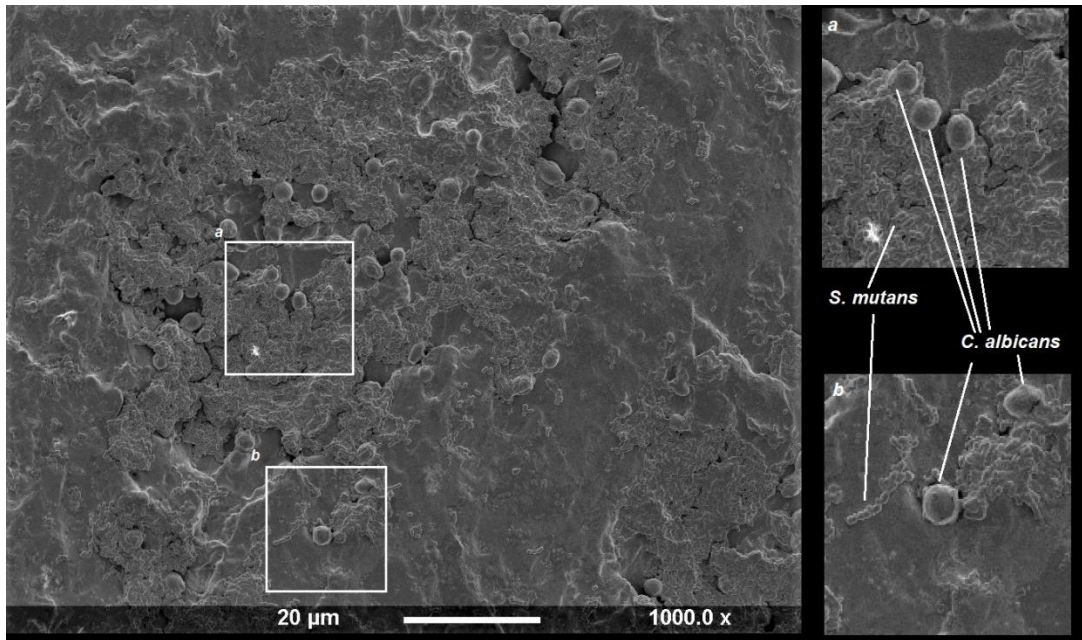


Figure 96 – 1000X magnification SEM image of an untreated 24 hour dual species biofilm of *C. albicans* and *S. mutans*. The big round cells correspond to *C. albicans* and the small ovoid shaped cells are *S. mutans* that grow in chains. The images on the side show the areas signalled where the cells can be seen in more detail and observe both cells living in close proximity.

The PMMA disks used in this experiment provided a further insight on the biofilm remaining in the disks after the experiment. SEM provided greater resolution and the images were taken at greater magnifications than in CLSM and enabled us to identify the individual cells comprising the biofilm.

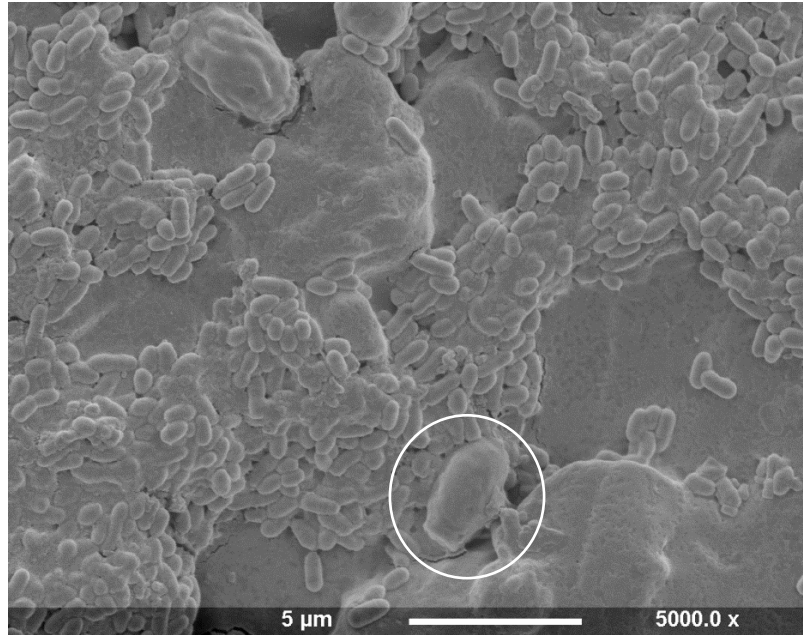


Figure 97 – 5000X magnification SEM image of an untreated 24 hour dual species biofilm of *C. albicans* and *S. mutans*. *C. albicans* (circled) in proximity of *S. mutans*. and the small ovoid shaped cells are *S. mutans* that grow in chains.

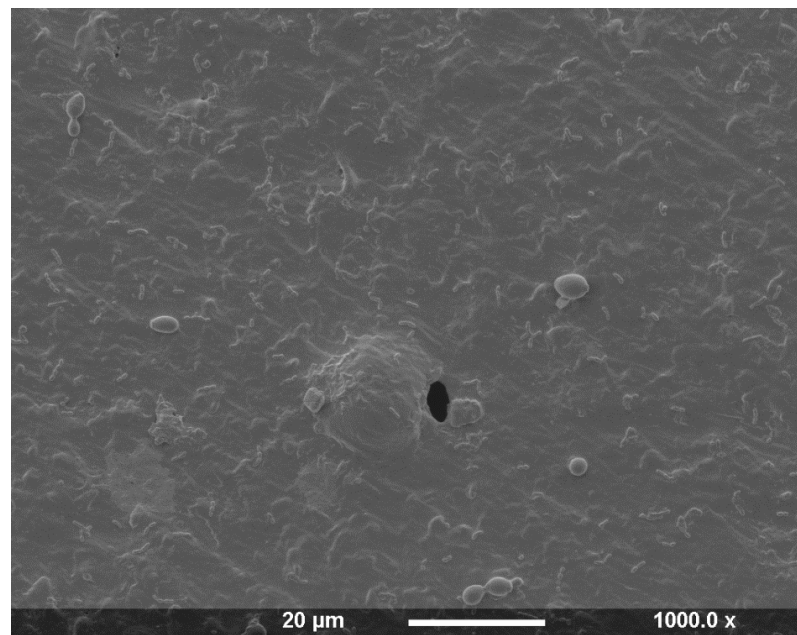


Figure 98 –1000X magnification SEM image of dual species biofilm of *C. albicans* and *S. mutans* after 10 minute challenge by the placebo tablet. The big round cells correspond to *C. albicans* and the small ovoid shaped cells are *S. mutans* that grow in chains.

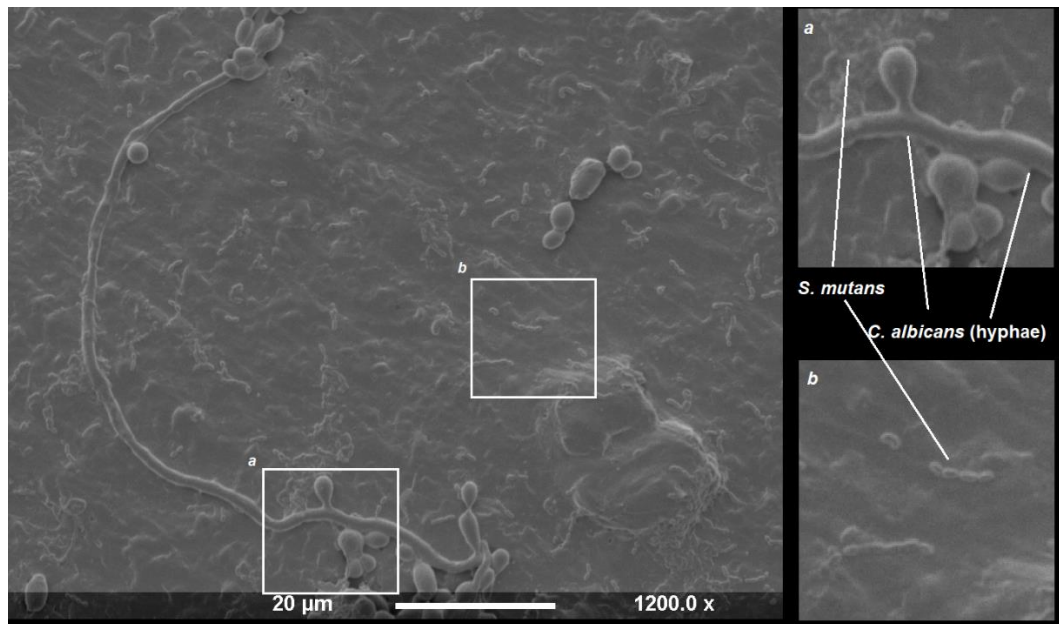


Figure 99 – 1200X magnification SEM image of 24 hour dual species biofilm of *C. albicans* and *S. mutans* after 10 minute challenge by Polident tablet solution. The big round cells correspond to *C. albicans* (some with hyphae) and the small ovoid shaped cells are *S. mutans* that grow in chains.

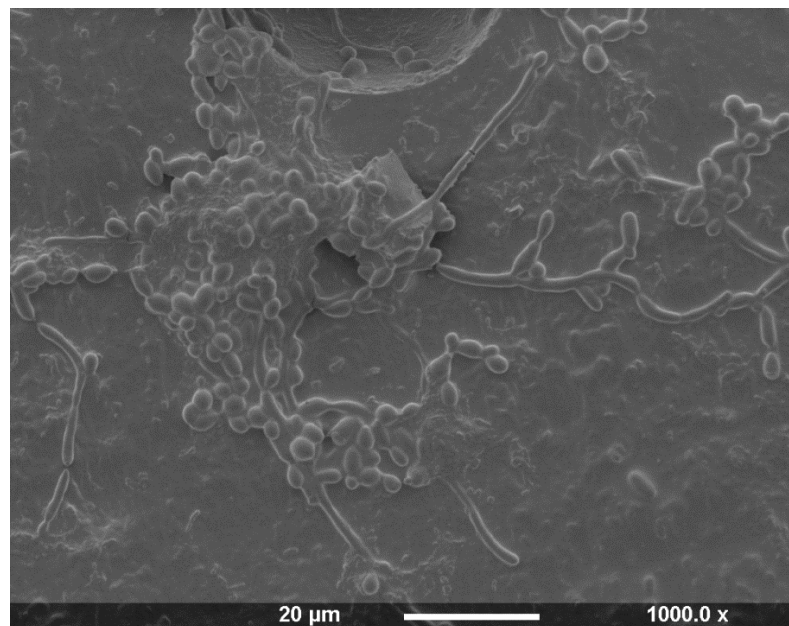


Figure 100 – 1000X magnification SEM image of 24 hour dual species biofilm of *C. albicans* and *S. mutans* after 10 minute challenge by the CA200 formulation. The big round cells, the elongated structures are presumably *C. albicans* growing in its hyphal form.

Observing the SEM images, Figures 95 to 100, of the dual species biofilm was observed that all the tested formulations removed a considerable amount of biofilm. At a first glance seems that the *C. albicans* was more resistant than *S. mutans* as evidenced by the viability tests with AlamarBlue.

The biofilms challenged by Polident and CA200, Figures 99 and 100 respectively, presented several *C. albicans* growing in the hyphal form. This is an indicator of a more virulent form also used by the organism to adapt to harsh environments^{23,149}. The only difference between placebo tablet (Figure 98) and Polident tablet (Figure 99) is the generation of ROS due to the addition of TAED and NaPC. The CA200 formulation with TAED and NaPC at a pH of 5.5 also generates ROS and presented *C. albicans* in the hyphal form.

The antimicrobial tests with Polident and ROS in solution showed that ROS are an effective antimicrobial agent with biofilm removal properties. *C. albicans* was harder to eliminate/remove in comparison with *S. mutans*.

4. Concluding Remarks and Future work

4.1 Concluding Remarks

NMR proved to be very resourceful method to study and follow the kinetics of a complex mixture of compounds, in this instance a denture cleanser tablet. Using $^1\text{H-NMR}$ this study dissected the reaction between TAED and NaPC by kinetically following its development at different temperatures and pH levels.

Tetraacetythylenediamine (TAED) reaction with H_2O_2 followed first order kinetics regarding the TAED and peracetic acid (PAA) decomposition also followed the same order of reaction meaning that these rates of the reactions only depend on the concentration of these reagents, TAED and PAA respectively. Temperature also played a crucial role, higher temperatures favoured decomposition of TAED and the generation and consumption of peracetic acid also increased.

The kinetics studies showed that the formation rates of the diacetythylenediamine (DAED) and acetic acid (AA) increased with temperature, using the reaction rates at different temperatures, the Arrhenius law was employed to determine the activation energies for the principal reactions taking place. The TAED decomposition into DAED had an activation energy of $29.8 \text{ kJ}\cdot\text{mol}^{-1}$ and the peracetic acid decomposition into acetic acid an activation energy of $73.2 \text{ KJ}\cdot\text{mol}^{-1}$. Considering that a single covalent bond has an energy between $150 - 400 \text{ kJ}\cdot\text{mol}^{-1}$ and a hydrogen bond $2 - 161 \text{ kJ}\cdot\text{mol}^{-1}$ it shows that the activation energy required for the reaction to take place will be easy to achieve¹²⁰.

When studying the pH of the Polident tablet and its ingredients it was observed that the Polident cleanser solution, prepared following the manufacturer indications, the pH of a solution left overnight gradually increased from 6.8 to 7.8. The single ingredient solutions prepared at the

tablet concentration presented the following pH values citric acid (CA) 1.65, polyethylene glycol 6.80, potassium monopersulfate (MPS) 1.93, sodium benzoate 7.01, sodium bicarbonate (NaHCO_3) 8.90, sodium carbonate (Na_2CO_3) 11.67, sodium lauryl sulfoacetate 7.37, TAED 6.35 and sodium percarbonate (NaPC) 10.73.

By combining both actives TAED and NaPC, at Polident concentration, the pH of the resulting solution started at 10.02 and slightly decreased overnight to 9.82. Note that a pH this high will not favour the spontaneous decomposition of PAA not resulting in the generation of singlet oxygen⁸⁰.

Not surprisingly only the combination of the 6 main ingredients TAED, NaPC, MPS, NaHCO_3 , Na_2CO_3 , CA at the tablet concentration resulted in solution with the optimal pH for the reaction to take place. Furthermore, the use of CA proved successful to lower the pH of the TAED/NaPC solution and allowed the reaction to take place in a successful way producing singlet oxygen as final product. Two novel formulations were developed, CA100 and CA200 with pH of 6.9 and 5.5 respectively. During the time lapse experiments the CA 100 formulation effectively mimicked the tablet solution pH while the CA200 maintained its pH between 5.5 and 5.7, as described in the literature, the optimal pH for the generation of singlet oxygen through PAA decomposition lies in the range of 5.5 and 8.2^{32,35,80,82}.

The fluorometric studies were successful in detecting the presence of ROS and singlet oxygen. By using dichlorodihydrofluorescein (DCFH) the generation of ROS other than H_2O_2 was detected. DCFH has the capability of being oxidized by several ROS¹²⁵. This proved useful to observe an increase in fluorescence by the generation of other species than H_2O_2 donated by NaPC. Since the half-life of singlet oxygen greatly increases in deuterium oxide (D_2O)¹⁵³ a parallel experiment was run alongside to ascertain the presence of singlet oxygen using DCFH in D_2O and in H_2O . As expected, the only the solutions containing NaPC showed a fluorescent signal indicating the presence of ROS and no difference between the D_2O

and H₂O. When TAED and NaPC were present in solution there was an increase in the fluorescent signal in comparison to the NaPC solution meaning that further ROS were being generated. In the solution made in D₂O the intensity of the fluorescence doubled a clear indicator of the presence of singlet oxygen in solution.

When using a placebo tablet manufactured as Polident tablet without TAED, NaPC and MPS on its own the tablet did not produced ROS hence no fluorescence was detected. The addition of NaPC produced a fluorescent signal that increased with addition of TAED as expected and previously observed. The solution made of placebo and MPS also generated a fluorescent signal showing that MPS had the capability of oxidizing DCFH. DCFH can be oxidized by strong oxidizers¹²³. Therefore, DCFH was not be suitable in pinpointing ROS generation within the tablet formulation.

Consequently, the fluorescent probe Singlet Oxygen Sensor Green specific to the detection of singlet oxygen was used for the remaining of the experiments in order to allow us to compare the generation of ROS and the novel formulations CA100 and CA200. Remarkably it was observed that the formulation CA200, with a pH at the lower limit of the optimal pH for singlet oxygen generation, showed an increase in the fluorescence signal in comparison with the CA100 formulation and Polident tablet solution. The CA100 formulation for the first minutes behaved in the same way as Polident showing a fluorescent signal with similar intensity but after 7 minutes the signal continued to increase while the Polident signal started to decrease. This was attributed to the presence of dyes within the formulation that can react with ROS decreasing the amount of singlet oxygen that can be detected. This can also be noted by the loss of coloration that occurs in the tablet solution while the placebo tablet solution does not show any colour change when the solution was left overnight at room temperature.

The combination of 1H-NMR and fluorometric assays proved to be a reliable method to screen and quickly test novel denture cleanser

formulations regarding the generation of ROS in situ and reactions taking place.

By decreasing the pH in solution the generation of singlet oxygen proceeded slower, mainly due to the poor dissociation of H₂O₂ at acidic conditions^{45,67} but on other hand it increased the amount of singlet oxygen generated showing that the pH of the final solution play a critical role in the effectiveness of the tablet.

Regarding the antimicrobial properties, the yeast *C. albicans*, as expected, was more resistant and harder to eliminate than *S. mutans* this can be attributed to the morphological differences between both organisms but also to the exceptional survival capabilities shown by yeasts and their ability to change its growth and propagation methods¹⁴⁹.

For the Kill Time, MIC/MBC determination three dental plaque model organisms were used, an early colonizer *S. mutans*, an intermediate colonizer *F. nucleatum* and a yeast *C. albicans* were challenged by Polident and placebo tablets. Since *F. nucleatum* as obligative anaerobe would struggle to thrive under experimental conditions, it was decided to not use this organism for the subsequent studies. Namely the single ingredient solutions MIC/MBC determination and biofilm studies. Both Polident and placebo, in the Kill Time showed good antimicrobial managing to eliminate *S. mutans* and *F. nucleatum* in one minute while for *C. albicans* took 10 minutes to be killed. This result was not surprising since the organisms tested were in the planktonic form and it is know that in this form they show less resilience towards antimicrobials and disinfectants^{6,154,155}.

Regarding the MIC/MBC determination for *S. mutans* and *F. nucleatum* 12.50% of the tablet solution contents were enough to eliminate both organisms and for *C. albicans* 50% and no MBC was detected at the tested concentrations showing that Polident tablet had only a bacteriostatic effect at the tested concentration. It is important to note that due to test limitations and the fact that a commercially available product is being used we can only start with a test concentration corresponding to 50% of the total

tablet contents. In the subsequent experiments all the solutions will be prepared at double of the tablet concentration to account for the initial dilution.

When the MIC/MBC ingredient solutions were tested against *C. albicans* only the citric acid solution, the CA200 and CA100, a combination of the six main ingredients (TAED, NaPC, NaHCO₃, Na₂CO₃, MPS and CA), the carbonates single solutions, NaHCO₃ and Na₂CO₃, and a solution containing TAED, NaPC, NaHCO₃ and Na₂CO₃ managed to eliminate this organism. *S. mutans* was eliminated by the solutions by the TAED, NaPC and CA solution, CA200, CA100, the six main ingredients combination, the TAED, NaPC, CA, NaHCO₃ and Na₂CO₃ solution, TAED, NaPC, NaHCO₃ and Na₂CO₃ and only one of the single ingredient solutions with NaHCO₃. TAED and NaPC on its own or together in solution managed to eliminate any of the organisms once more validating the pH requirement and PAA spontaneous decomposition for antimicrobial activity. The pH of the tested solutions ranged from 1.64 to 11.67 and no trend was observed suggesting that the pH on its own exerted any antimicrobial activity.

For the biofilm studies, it was determined that the optimal cell concentration to produce a detectable using AlamarBlue and a 24 hours incubation period in a MBEC device coated with HA for the organism *C. albicans* was 10^7 - 10^8 CFU/ml the same value was used to grow the *S. mutans* biofilm. In the PMMA disks the biofilms grown were dual species biofilms comprising both species, inoculated at the same concentration, and were used for the visualization experiments. Building up from the previous experiments only the following solutions were tested the CA200, Polident tablet and Placebo.

In the testing using the MBEC device the placebo tablet that, showed significant antimicrobial activity in the planktonic test (MIC), such activity was only observed towards *S. mutans* were 100% of the concentration managed to remove the majority of the biofilm leaving around 4% attached to the pegs, concerning *C. albicans* 50% of the tablet solution only managed to remove

around 53% of the biofilm. The Polident tablet contents at 50% concentration were enough to remove both single species biofilms of *S. mutans* and *C. albicans*, note that for the MIC 50% were also necessary to arrest the growth of *C. albicans* while *S. mutans* was eliminated with 12.5% of the tablet contents in the planktonic form. The novel formulation CA200 managed to virtually remove all of the *S. mutans* biofilm at 100% and 50% concentration leaving 3% and 7% of biofilm respectively. When challenging *C. albicans* 100% of its total contents removed all biofilm, 50% and 25% concentration left 35 and 89% of biofilm respectively.

The visualization experiments agreed with the previous antimicrobial tests. The confocal imaging showed that the Placebo tablet without actives removed biofilm to some extent, but as also evidenced by the viability assay the great majority of the remaining biofilm was alive. The biofilms challenged by Polident and CA200 did not present a significant difference between them and virtually all of the remaining cells were dead, and the biofilm was considerably thinner than the results obtained from the Placebo tablet. SEM imaging further scrutinized the remaining biofilm, after being challenged, and showed that after the treatment with ROS most of the remaining organisms were *C. albicans* in the hyphal form known for being a virulence and survival trait of *C. albicans*¹⁴⁹.

By comparing all the results and taking into account that the placebo tablet does not have ROS in its formulation nor the capability of producing it there is a clear indication that ROS play a significant part in the antimicrobial properties observed by the denture cleanser formulation.

4.2 Future work

This project elucidated the reaction mechanisms behind Polident, a denture cleanser tablet, leading to the generation of ROS. ROS are known for its antimicrobial and bleaching properties as discussed throughout this work.

Regarding the methodology, used the $^1\text{H-NMR}$ proved to be an extremely useful tool to follow the reagents and products within the formulation but due to instrument limitations the initial stages of the reaction could not be measured. Hence, in the immediate continuation of this work the method must be optimized to address this issue. This could be resolved by drastically reducing the reagents concentration and in solution to reduce the bubble formation and effectively reduce the time necessary to acquire the first measurements.

The combination of $^1\text{H-NMR}$ and fluorimetry proved to be a reliable method to quickly screen a formulation and identify the chemical reactions taking place and pinpoint if ROS are being generated with some optimization this method will be standardized and employed in the screening of novel and existing formulations.

In this work Polident and ROS proved to be an excellent antimicrobial effective in the removal of biofilms, but no studies were made regarding the mechanisms on how ROS were effectively killing bacteria and damaging the biofilm. Therefore, it would be interesting to perform a follow up study observe such effects in more detail. Confocal microscopy could be used to give some insights into this question. By using a suitable combination of chromophores targeted at different structures of the biofilm it would be possible to observe which components of the biofilm are being removed^{21,156}. Also the recent developments in microscopy, specifically in the transmission electron microscopy field employing novel liquid cells and high-resolution cameras can present us with real time images of biofilm structures and membrane damage caused by ROS^{78,157}.

The biofilm models used were quite simple in comparison with the denture/oral biofilm that can have around 200 species^{14,106,158} using a more complex model would definitely improve the quality of the results obtained and present a more robust challenge the efficacy of ROS and bring the testing closer to a real life scenario. Current research being conducted by Infection and Immunity Research Group of the University of Glasgow Dental School is developing standardized biofilm models that model different stages of periodontal disease²¹. It would be interesting to collaborate with their group and test the developed formulation against a more challenging biofilm.

It is widely acknowledged that poor denture hygiene will lead to health complications not only at an oral level but also other systems within the human body can be affected such as digestive, respiratory and circulatory systems^{19,111,112,159}. The World Health Organization, in 2002, recognized in the World Oral Health Report reported that oral health issues can also be the cause of confidence problems that can give rise to mental health issues^{4,5}. Denture wearers are nearly “invisible” in our society and the use of dentures is on the increase therefore mounting the demand for more effective cleaning solutions and methods to improve oral health⁴ increasing the need for more research and development in denture cleansers.

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Appendix - Microbiology Medias

Brain Heart Infusion (BHI) Broth

Reagents

Brain Heart Infusion Powder (BHI) (Oxoid, UK)

Distilled water

Preparation

18.5 g of the BHI dry powder were dissolved in 500 ml of distilled water and subsequently sterilized by autoclaving (121 °C, 15 minutes). Once prepared the media was stored at room temperature for up to two weeks.

Composition:

| | |
|--------------------|----------|
| Brain Extract | 12.5 g/L |
| Heart Extract | 5.0 g/L |
| Peptone | 10.0 g/L |
| Glucose | 2.0 g/L |
| Sodium Chloride | 5.0 g/L |
| Disodium Phosphate | 2.5 g/L |

Columbia Blood Agar

Reagents

Columbia Agar Powder (Oxoid, UK)

Distilled water

Defibrinated Horse Blood (EO Labs, UK)

Preparation

36.5 g of the Columbia agar dry powder were dissolved in 855 ml of distilled water and subsequently sterilized by autoclaving (121 °C, 15 minutes) and allowed to cool down in a water bath down to 50 °C.

Subsequently 45 ml (5% of the total volume) of horse blood at room temperature was mixed with the agar and the resulting mixture poured in 90 mm petri dishes and allowed to cool down.

Once set the plates were stored at 4 °C and used within 1 month.

Composition:

| | |
|-----------------|----------|
| Casein Peptone | 12.0 g/L |
| Meat Peptone | 5.0 g/L |
| Sodium Chloride | 5.0 g/L |
| Beef Extract | 3.0 g/L |
| Yeast Extract | 3.0 g/L |
| Cornstarch | 1.5 g/L |
| Agar | 13.5 g/L |

Phosphate Buffer Saline Solution

Reagents

Phosphate Buffer Saline tablets (Dulbecco A) (Oxoid, UK)

Distilled water

Preparation

5 pills of the PBS were dissolved in 500 ml of distilled water and subsequently sterilized by autoclaving (121 °C, 15 minutes). Once prepared the solution was stored at room temperature for up to two weeks.

Composition:

| | |
|--------------------------------|----------|
| Sodium Chloride | 8.0 g/L |
| Potassium Chloride | 0.2 g/L |
| Di-sodium Hydrogen Phosphate | 1.15 g/L |
| Potassium Dihydrogen Phosphate | 0.2 g/L |

Artificial Saliva

Reagents

Lab Lemco Powder (Oxoid, UK)
Yeast Extract (Oxoid, UK)
Protease Peptone (Oxoid, UK)
Mucine from porcine stomach (Oxoid, UK)
Sodium Chloride (NaCl) (Sigma-Aldrich)
Calcium Chloride (Ca₂Cl) (Sigma-Aldrich)
Potassium Chloride (KCl) (Sigma-Aldrich)
Urea (Sigma-Aldrich)
Sucrose (Sigma-Aldrich)

Preparation

The following ingredients dissolved in 500 ml of distilled water and subsequently sterilized by autoclaving (121 °C, 15 minutes). Once prepared this media was stored at 4 °C for up to one month.

| | |
|-----------------------------|---------|
| Lab Lemco Powder | 0.5 g |
| Yeast Extract | 1 g |
| Protease Peptone | 2.5 g |
| Mucine from porcine stomach | 1.25 g |
| NaCl | 0.175 g |
| CaCl ₂ | 0.1 g |
| KCl | 0.1 g |

A solution of 40% urea in distilled water and a solution of 50% sucrose were also prepared, filter sterilized and stored at 4 °C.

Prior use 125 µL/100ml of the urea solution and 2ml/100ml of the sucrose solution were mixed with the AS to attain a content of 0.05% and 1% respectively in the final AS solution.

SEM Specimen Preparation

Reagents

Sodium Cacodylate (Sigma-Aldrich)

1M Hydrochloric Acid (HCL) (Sigma-Aldrich)

1M Sodium Hydroxide (NaOH) (Sigma Aldrich)

Paraformaldehyde (Sigma-Aldrich)

50 % Glutaraldehyde (Sigma-Aldrich)

Ethanol 99.9% (Sigma-Aldrich)

Hexamethyldisilazane (HDMS) (Sigma-Aldrich)

Distilled water

Fixative Solution

Cacodylate Buffer 2M

8.56 g of sodium cacodylate were dissolved in 200 ml of distilled water and the pH adjusted with HCL to 7.4.

Modified Karnovski Fixative (4% paraformaldehyde, 2.5% glutaraldehyde)

1 g of paraformaldehyde was dissolved in 20 ml of warm distilled water and a few drops of NaOH were added until the solution become clear and allowed to cool down. Then 2.5 ml of the glutaraldehyde solution and 25 ml of the 0.2 M cacodylate buffer were added to the solution and the pH adjusted using NaOH and HCl to 7.4.

The solution was stored for later use at 4 °C.

Dehydration

Three solutions of ethanol were prepared by diluting ethanol 99.9% with distilled water. The solutions prepared were 50%, 70%, 90% and 99.9% and stored at room temperature.

Drying

A fresh solution of ethanol and HDMS (1:1) was prepared just before being used and an aliquot of pure HDMS was stored away for the final step.