# FACTORS INFLUENCING SORPTION, SOLUBILITY AND CYTOTOXICITY OF A HEAT CURED DENTURE BASE POLYMER

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Each member of my family, for their unique, individual contribution:  I love you.

# DECLARATION

I declare that the following thesis "*Factors influencing sorption, solubility and cytotoxicity of a heat cure denture base polymer*" is my own work, that it has not been submitted before for any degree or assessment at any other university, and, that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

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**Magdalena Aletta Engelbrecht**

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# SUMMARY


**Title:**

Factors influencing sorption, solubility and cytotoxicity of a heat-cure denture-base polymer.

**Key words:**

Sorption, solubility, cytotoxicity, denture base.

**Objectives:**

Substances leaching from denture- base polymers have been associated with cytotoxicity and allergic reactions. This study examined the effect of polishing, mixing ratios, water immersion temperatures and different thicknesses on the sorption and solubility of a heat-polymerized, denture-base polymer. The effect of different water immersion temperatures on the flexural strength of the denture base, was tested as well. The next component of this study, is the testing of the most significant sorption and solubility findings on *in vitro* cell viability.

**Materials and Methods:**

Disc shaped specimens from a heat-polymerized, denture-base polymer ( Vertex®) were prepared, based on ISO 1567 specifications for sorption and solubility testing, following the manufacturers' instructions. The following tests were performed: 1) Sorption and solubility of two groups (n = 12 each) of

polished and unpolished discs were established and compared by means of the Mixed procedure; 2) Sorption and solubility of three groups (n = 12 each) with different mixing ratios were compared by means of the Mixed procedure; 3) Four groups (n = 14 each) were immersed in water at different temperatures, sorption and solubility were compared by means of pairwise comparison and the Median test; 4) Specimens with different thicknesses (n = 36) were compared, again, by means of pairwise comparison and the Median test; 5) To test the influence of different water-soaking temperatures on the flexural strength of the disc, strips were prepared from the disc used in test no. 3. The flexural strength was compared, by means of the Median test; 6) To test the influence of no post-polymerization treatment, polishing and water immersion on the cytotoxicity of mouse fibroblast cells, (n = 9) for each test group, were prepared. A preliminary test was performed beforehand, over a period of 24 hours, up to a maximum period of four weeks. The Balb/c 3T3 mouse fibroblast cells were cultured and incubated for 24 hours in Eagles medium. Eluates prepared from the disc and medium without any disc (control) replaced the medium. Cytotoxicity was assessed by MTT-assay. Optical density values were obtained at 24 and 48 hour intervals. The data was analyzed by means of the Means procedure.



**Results:**

In the entire thesis, the data was analyzed using SAS on a 0.01 probability level. Between polished and unpolished groups, no significant difference in water sorption ($p > 0.01$) was found, but there was a difference in solubility ($p < 0.01$). Different mixing ratios did not alter sorption ($p = 0.34$) or solubility ($p = 0.68$).

However, a difference ($p<0.01$) in sorption and solubility was found among the different temperature and thickness groups. Soaking the denture base in water at different temperatures did not alter its flexural strength ($p = 0.48$). Cell viability levels were noted in all the experimental groups in the MTT assay test. The analysis was a two-factor study, with one factor being the group, and the other, being time. The interaction between these factors was found to be significant, indicating that the effect of the groups varied by time (and vice versa).

**Conclusion:**

The processes of the soaking in warm water and the polishing of a denture-base polymer, reduce its solubility. Therefore, it is recommended that dentures are soaked in warm water before polishing. Within the limits of this study, the mixing ratios may be changed without affecting sorption or solubility. As solubility increases within the increasing denture-base thickness, it is recommended that unnecessarily thick dentures be avoided.



Short- and long-term exposure to eluates of a PMMA, has a negative effect on cell viability. For water-stored and polished discs, this effect is time-dependent, with a higher viability for 48 hours', than for 24 hours eluates. Polishing is associated with lower solubility. At 24 hours, the polished discs, indeed, had a lower cytotoxic effect than the untreated discs: it may be recommended that dentures be polished on the fitting surface as well.

The cytotoxic potential of PMMA-eluates appears to fluctuate over time.

# CHAPTER 1

# INTRODUCTION, PROBLEM STATEMENT AND LITERATURE REVIEW

## 1.1. Introduction

Polymethyl methacrylate (PMMA) resin has a wide variety of dental, medical and industrial applications. In dentistry, it is frequently used in the fabrication of removable complete and partial dentures, interim fixed partial dentures, splints and removable orthodontic appliances. Its use in dentistry is widespread because of its versatility, ease of use and its various shade options that match the shades of oral tissues.



## 1.2. Definitions

Allergy

> "Allergy is a hypersensitivity reaction to an allergen which is enhanced by repeated exposure". (Hochman & Zalkind, 1997).

Immediate hypersensitivity allergic reaction

> "Immediate hypersensitivity response generally occurs within 12 minutes of an antigen challenge. These reactions are mediated by T-cells and monocytes/macrophages rather than by antibodies". (www.emedicine.com).

Delayed hypersensitivity allergic reaction

"Delayed hypersensitivity reactions are inflammatory reactions initiated by mononuclear leukocytes. The term 'delayed' is used to differentiate a secondary cellular response, which appears 48 - 72 hours after antigen exposure". (www.emedicine.com).

Cell death

Apoptosis

"Apoptosis is an active and physiological process characterized by cell shrinkage, detachment from neighbouring cells, condensation of nuclear chromatin followed by nuclear fragmentation, and preservation of the structural integrity and most of the functions of the plasma membrane and of the cellular organelles". (Majno & Joris, 1995).



Necrosis

"Necrosis is a passive and degenerative process occurring as a result of the cell's exposure to gross injury. It is characterized by mitochondrial swelling, dissolution of the nucleus, rupture of the plasma membrane and the release of the cytoplasmic constituents. Necrosis triggers an inflammatory reaction in the tissue and often results in scars". (Majno & Joris, 1995).

Cytotoxicity

"Cytotoxicity means toxic to cells, cell-toxic or cell-killing. It is caused by any agent or process that kills cells. Chemotherapy and radiotherapy are forms of cytotoxic therapy as they kill cells. The prefix cyto- denotes cell. It comes from the Greek *kytos* meaning hollow, as a cell or container. Toxic is from the Greek *toxikon* = arrow poison". (www.medicinenet.com).

Contact dermatitis

"Contact dermatitis is an inflammation of the skin caused by direct contact with an irritating or allergy-causing substance (irritant or allergen)". (www.medlineplus.gov).



Irritant contact dermatitis

"Irritant contact dermatitis is the most common type of contact dermatitis. It involves inflammation resulting from contact with acids, alkaline materials such as soaps and detergents, solvents, or other chemicals. The reaction usually resembles a burn". (www.medlineplus.gov).

Allergic contact dermatitis

"Allergic contact dermatitis, the second most common type of contact dermatitis, is caused by exposure to a substance or material to which a person has become hypersensitive or allergic. The

allergic reaction is often delayed, with the rash appearing 24 - 48 hours after exposure". (www.medlineplus.gov).

Dimensional stability

"Dimensional stability is defined as the ability of a substance or part thereof to retain its shape when subjected to varying degrees of temperature, moisture, pressure, or other stress". (www.about.com).

Denture base

"The denture base is that part of the denture which rests on soft tissue foundations and to which teeth are added". (ISO 1567).



Eluate

"An eluate is the solution of solvent and dissolved matter resulting from elution". (www.thefreedictionary.com).

Flexural strength

"The transverse strength, modulus of rupture, or flexural strength is the strength of a material in bending, the resistance to fracture". (www.thefreedictionary.com).

Polymethyl methacrylate resin

"A polymethyl methacrylate (PMMA) resin is polymerized from methyl methacrylate (MMA) by an additional polymerization reaction. This

reaction can be activated by heat or chemical activators such as dimethyl-p-toluidine". (Phillips, 1991).

Heat-polymerized polymer

"A heat-polymerized polymer is a product requiring application of verifiable temperatures above 65ºC to complete polymerization". (ISO 1567).

Solubility

"The solubility of a substance is its concentration in a saturated solution. The solubility is sometimes called "equilibrium solubility" because the rates at which solute dissolves and is deposited out of solution are equal at this concentration". (www.diracdelta.co.uk).



Sorption

"Sorption is described as the assimilation of molecules of one substance by another material in a different phase. Sorption consists of *ad*sorption and *ab*sorption. Adsorption is the adhesion of a chemical species onto the surface of particles. Absorption is the process by which atoms, molecules, or ions enter a bulk phase (liquid, gas, solid). Absorption differs from adsorption, since the atoms/molecules/ions are taken up by the volume, not by surface". (www.about.com).

### 1.3. Problem statement and purpose of the study

The nature of the raw ingredients for the manufacturing of appliances from acrylic resin materials in dentistry, depends on the type of polymerization. For auto- or heat-polymerization systems, the material is often supplied in a powder/liquid combination. Although it is recommended that manufacturers' instructions are followed, mixing ratios are modified in an effort to increase working time and to allow for a larger number of flasks to be filled simultaneously.

Different brands of PMMA resins for denture bases have different recommended polymerization cycles. Traditionally, dentures are polymerized slowly, overnight, at temperature below water boiling point, while newer brands have shorter cycles at higher temperatures, some as short as 20 minutes, in boiling water. Again, manufacturers' instructions are not always followed and polymerization cycles are shortened to save time.



Finished dentures need to be polished before delivery to the patient. Often, at delivery or follow-up visits, dentures are adjusted. This alters the polished surface leaving it rough. Due to lack of time or equipment, dentures are not always re-polished after adjustments. In some instances, the patients may even adjust the dentures at home, leaving an unpolished surface. Unpolished surfaces may influence the sorption and solubility properties of the dentures.

Dentists advise patients to remove dentures at night and place them in a glass of water. Water immersion temperatures may have an influence on dentures' sorption and solubility properties, as well as on the strength of a denture.

The purpose of this study was to

a) investigate the influence of mixing ratio, post-polymerization water immersion temperatures, polishing and different thicknesses on the sorption and solubility of a denture-base resin;

b) study the effect of different water immersion temperatures on the flexural strength of a denture- base resin; and

c) establish if the eluate of a denture- base resin has a cytotoxic effect.



### 1.4. Literature review

#### 1.4.1. The need for dentures

After tooth loss, a dental prosthesis restores appearance and function. In South Africa, removable dentures appear to be a popular choice to replace missing teeth. Hartshorne & Carstens (1991) assessed the need for dentures by looking at the applications submitted to the Department of Health in the Western Cape region during a two-year period. From the 4573 applications almost 60 per cent of the applicants had been edentulous for 1-10 years, and the greatest demand was for full upper and lower dentures. This shows that, in the Western Cape, the need for dentures to replace missing teeth have not been met in the 1980's. The need for dentures is expected to continue throughout life for many patients from the Western Cape population.

### 1.4.2. Denture base polymers

MMA is an organic compound with the formula $CH_2=C(CH_3)COOCH_3$ (Figure 1). Methacrylates easily form polymers because the double bonds are very reactive (Figure 1). In industry, PMMA is a thermoplastic material often used as a light or shatter-resistant alternative to glass and an economic alternative to polycarbonate when extreme strength is not necessary. Additionally, PMMA does not contain the potentially harmful bisphenol-A found in polycarbonate.

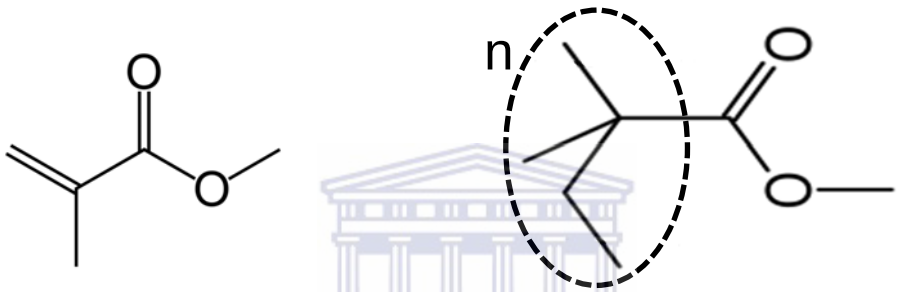


*Figure 1: On the left is the formula for methyl methacrylate. On the right is the skeletal formula for (polymethyl) methacrylate. n indicates the number of polymer bonds (Images of simple structural formulae are ineligible for copyright. Images sourced and modified from Wikipedia).*



PMMA resins have been used for the manufacturing of dentures for more than 60 years (Kedjarune *et al.,* 1999). The material is readily available, it is versatile, easy to repair and does not require expensive laboratory equipment.

Most PMMA resins for dentures are heat-polymerized, but other methods of polymerization exist. The ISO proposes the following classification of denture base polymers (ISO 1567 (E): 1999):

Type 1: Heat-polymerized polymers

Class 1: Powder and liquid

Class 2: Plastic cake

Type 2: Auto-polymerized polymers

Class 1: Powder and liquid

Class 2: Powder and liquid pour-type resins

Type 3: Thermoplastic blank or powder

Type 4: Light-activated materials

Type 5: Microwave-polymerized materials



### 1.4.3. Sorption

Food, fluids and even denture cleansers may stain and discolour dentures (Ma *et al.*, 1999). This is the result of sorption, a process of adsorption and absorption (Keyf & Etikan, 2004). Keyf & Etikan (2004) describe adsorption as a process by which molecules, colloids and particles adhere to a surface by physical action, without chemical action. Absorption on the other hand is a process by which atoms, molecules, or ions enter a bulk phase (www.about.com). Polar properties of the resin molecules facilitate this water absorption.

The sorption and release of water from the material cause dimensional instability and create internal stresses that may result in crack formation and fatigue fracture of a denture (Beyli & Von Fraunhofer, 1981). Hiromori *et al*. (2000) reported that water absorption increases the volume of material and makes the material more

plastic. Higher temperatures promote this plasticity. Expansion due to water sorption compensates for polymerization shrinkage (Ristic & Carr, 1987). Within limits, this compensation should have a positive influence on the fit of a denture. Dixon *et al.* (1992) compared the linear dimensional changes of three denture base materials (heat-, rapid-heat- and light-polymerized resins) during processing and after storing the materials in water for 30, 60, and 90 days. It was found that the rapid-heat-polymerization material exhibited the least shrinkage, while the heat-polymerized resin exhibited the most shrinkage. However, the difference between the groups was not significant. After 90 days of water storage, the only resin that exhibited nett shrinkage from the processed state, was the rapid-heat-polymerization group. All of the expansion or shrinkage changes were so small that they were not statistically significant and should not be clinically detectable. However, Monfrin *et al*. (2005) studied the dimensional contour variations of specimens shaped like dentures, before and after storage in water for 42 days. Significant differences for both the maxillary- and the mandibular-shaped bases were found. They concluded that water sorption had an important effect on the contour of the prosthetic bases. The methodology of the latter study relates more to the clinical setting by making use of denture shapes. The shape, thickness and size of a denture base all have an effect on dimensional changes (Sadamori *et al.*, 1994).



The flexural strength of denture bases is an important mechanical property. The longevity of dentures depends largely on the transverse strength of the acrylic resin. According to Dhir *et al.* (2007), the physical and mechanical properties of denture base resins are adversely affected by extensive absorption of water: it may

result in a plasticizing effect after prolonged use. Water absorption may prevent the intermeshing of polymer chains, causing them to be progressively more mobile, resulting in the relaxation of built-up internal polymerization stresses (Ristic & Carr, 1987). Water sorption by acrylic resins contributes to dimensional instability and fatigue, which may lead to crack formation and, subsequently, to the fracturing of the denture (Beyli & Von Fraunhofer, 1981). Paragraph 1.4.5. will deal with the effect that immersion in water may have on the transverse strength of denture base resin.

Most denture base polymers have varying amounts of cross-linking agents added to the mixture. These cross-linking agents enhance the conversion of monomer into polymer (Dhir *et al.*, 2007). Jagger & Huggett (1990) state that the presence of cross-linking agents has a small effect on water sorption.



Doğan *et al.* (1995) reported a parallel reaction between the level of MMA and water absorption. As MMA diffuses, water is replaced into the voids of the polymer mass. With high levels of MMA, more voids are left after the leaching of MMA and water intake is higher (Kalachandra & Turner (1987) in Doğan *et al.* (1995)).

### 1.4.4. Solubility

PMMA denture base resins are regarded as insoluble in water and in most other fluids that may be present in the oral cavity (Miettinen *et al.*, 1997). However, substances within the PMMA matrix may leak from the polymer matrix.

Initiators, plasticizers and free monomer are all soluble materials present in denture base resins (Knott *et al.*, 1988). Concerns have been raised regarding the sensitization and potential toxicity of some of these components leaking from denture base resins, in particular, mono-MMA.

Loss in weight of a specimen is regarded as a measure of the soluble material present and of the soluble material leaking from the specimen. Knott *et al.* (1988) reported that a positive correlation between residual monomer and weight loss was observed in solubility tests. Using high performance liquid chromatography, a positive correlation between residual monomer content and monomer leaching from denture base polymers in water has been established (Vallittu *et al*., 1995).

Although most compounds are released within the first few days of use, the release may continue for years (Sadamori *et al*., 1992; Vallittu *et al*., 1995).



In terms of this prolonged release of monomer, Lung & Darvell (2005) dealt with an interesting concept that is seldom, if ever, mentioned in the literature, i.e. the equilibrium between polymerization and depolymerization (MMA↔PMMA). This equilibrium might not be reached in an open system, such as dentures where MMA is lost to the surroundings. Trying to re-establish equilibrium, creates a constant source of MMA through depolymerization and, according to Lung & Darvell (2005), this cannot be eliminated. In fact, they are very critical of the lack of understanding that researchers have of this concept.

Not much literature could be found linking solubility and the thickness of denture bases, but Cucci *et al.* (1998) reported that solubility decreased proportionally to the thickness of the specimen.

More information in terms of the release of potentially hazardous substances from denture base resins will be provided in paragraph 1.4.12.

### 1.4.5. Immersion in water and transverse strength

PMMA denture bases need thickness to provide strength to a prosthesis which is expected to withstand masticatory forces for many years. Dentists often advise patients to store their dentures in water when not in use. The strength of a denture base should not be affected by habits such as either storing dentures in water or by using denture cleansers.

As mentioned earlier, PMMA absorbs water. Absorbed water acts as a plasticizer of PMMA and reduces its flexural strength (Dixon *et al.*, 1991; Miettinen *et al*., 1997). Dixon *et al.* (1991) investigated the influence of water immersion on the transverse strength of a high-impact heat-polymerized resin, a rapid heat-polymerized resin, and a light-activated denture base material. Their results indicated that the light-activated resin had the lowest transverse strength of the three materials. However, water storage for 30 days didn't negatively influence its strength while the strength of the other two materials decreased with water immersion. The difference in chemical structure between the heat- and light-activated resins was used as an explanation for the difference in behaviour after water immersion.

### 1.4.6. Polishing

Ulusoy *et al.* (1986) emphasized the importance of a polished denture surface. They postulate that smooth and highly-polished dentures are important for patient comfort, aesthetics, denture hygiene and prosthesis longevity. Surface roughness on denture bases promotes the adhesion of micro-organisms and the formation of plaque. Kuhar & Funduk, (2005) examined how different polishing systems would affect the surface roughness of denture base acrylic resins. They found the highest surface roughness on surfaces finished with a tungsten carbide bur and the lowest surface roughness after the product had been polished with a lathe and polishing paste.



Besides the advantages of a polished denture surface mentioned in the previous paragraph, researchers have been investigating if surface treatments also reduce the leaching of substances from the denture base into the oral cavity. Tsuchiya *et al.* (1993) wanted to know if an ultraviolet light-activated coating reduced the amount of MMA and formaldehyde leaching from auto-polymerized denture resins. Therefore, coated and uncoated disks were immersed in artificial saliva. The concentration of the leached substances in the saliva was established by means of high-performance liquid chromatography and flow-injection analysis. Coated specimens released significantly less MMA and formaldehyde than uncoated specimens.

Vallittu (1996) examined whether a polishing process and a light-polymerized varnish would influence the residual monomer release from an auto-polymerized

denture resin in water. The author demonstrated that both the light-polymerised coated resin and the conventional polishing of the denture surface were effective in reducing the release of monomer. These two studies proved that surface treatment may have an influence on the leaching of substances from resins. Although both studies used auto-polymerized resins, it is presumed that the same effect would exist if these surface treatments were applied to heat-polymerized resins. However, no literature could be found dealing with the effect of polishing of a heat-polymerized denture base resin on sorption and solubility.

### 1.4.7. Mixing ratios

Producers of PMMA resins dentures each recommend different powder/liquid mixing ratios for their products. These ratios may differ for each brand. Mixing ratios may also be modified by laboratory technicians in order to manipulate handling properties. Of particular concern, is the incorporation of more liquid (monomer) to extend working time.

Kedjarune *et al*. (1999) investigated the amount of residual monomer content and MMA released into saliva, after processing. They confirmed that the amount of residual monomer is not only dependent on the powder/liquid ratio, but also on the type of polymerization (auto- or heat-polymerization), and the processing method. The acrylic resin with the lowest residual monomer content also released the smallest amount of MMA. However, resins with higher monomer content, did not necessarily release more MMA. When MMA, in the same range of concentration as the MMA found in the test saliva, was tested on cell cultures, cytotoxicity was noticed.

It is speculated by Jerolimov *et al.* (1985) that the choice of polymerization cycle has a greater influence on the MMA level in PMMA than the initial powder/liquid mixing ratio.

### 1.4.8. Water storage temperature

Since residual monomer is identified as a hazardous substance in denture base polymers, methods of reducing its content before delivery to a patient, have been considered. One of the easiest and cheapest methods of doing so would be soaking a denture in water, before delivering it to the patient. Several studies confirmed this and also confirmed that higher water temperatures have an influence on diffusion rates (Ruyter & Oysaed, 1982).



Tsuchiya *et al*. (1994) concluded that the residual monomer content of denture base materials is lowered to a quarter of the initial value if the product is immersed in water at 50ºC for one hour after polymerization, compared to not soaking.

Vallittu *et al*. (1995) studied the residual monomer in an auto- and heat-polymerized denture base resin during water immersion, at 22ºC and 37ºC, over time. Their results showed that an increased water-immersion temperature enhanced the diffusion of MMA into the water most significantly on the first day of immersion. Until day 14 of water immersion, there is a noticeable difference in the amount of monomer released into the water. Thereafter the monomer content in the two test groups remained stable up to day 30 of immersion. Therefore, they

confirmed that storing a dental acrylic resin in distilled water at 37 °C is a simple, but effective, method of reducing its residual MMA content.

### 1.4.9. Denture base thickness

The influence of the thickness of acrylic resin plates on the residual monomer content was examined by Sadamori *et al*. (1994) by means of gas liquid chromatography. They found that the levels of residual monomer were influenced by the processing methods and thickness of the specimens. Thinner specimens had a higher level of MMA content than thicker specimens. This correlates with the findings of Fletcher *et al*. (1983) who also found that thicker specimens have a lower MMA content than thin specimens using gas liquid chromatography. Austin & Basker (1980) explain that more heat is developed in thicker specimens during heat-polymerization, resulting in a higher degree of monomer conversion with a corresponding reduction in residual monomer. However, Sadamori *et al*. (1994) found that the monomer content was not influenced by the location within the specimen.



A later study by Sadamori *et al.* (1997) examined the influence of thickness on changes in linear dimensions, on warping, and on water sorption in a denture-base resin. They found that changes were again influenced by both the processing method and the thickness of the specimens. Thicker specimens absorbed more water and took longer to reach stable dimensions compared to the thinner specimens. However, these researchers did not follow ISO procedures and did not take into account the loss of substance during water immersion. They also did not

report their results per-unit-volume as ISO requires, making comparison among different thickness groups problematic.

### 1.4.10. Allergies

Dental staff and patients are at risk of developing sensitization and allergic reactions to dental materials. Vilaplana *et al.* (1994), in Pfeiffer & Rosenbauer (2004) reported that contact dermatitis may be caused by cobalt, nickel, beryllium and MMA.

Kanerva & Estlander (1993) in Pfeiffer & Rosenbauer (2004) report that dental technicians are at high risk for developing allergies to denture base resins. Murer *et al*. (1995) in Pfeiffer & Rosenbauer (2004) reported that this risk is 8 times higher among dental technicians than among the general population.

According to Gawkrodger (2005), patients undergoing dental treatment, are exposed to a wide range of potential allergens, but adverse events seem infrequent. He says that symptoms or signs of stomatitis, burning, tingling, cheilitis, oral lichenoid lesions, and lip and facial swelling may be related to the use of dental products.

Most allergic reactions are generally delayed, or of a dermal type, among dental patients, and manifest themselves in the form of contact dermatitis among dental personnel (Hensten-Pettersen & Jacobsen, 1991). They suggest that sensitization is caused by repeated contact with either allergy-inducing dental materials or by components found in jewellery, perfume or household products. Gawkrodger (2005) writes: “The main allergic reactions found in patients include contact

allergy to metals, cosmetics, food additives, flavours and acrylates, and immediate type allergy to latex".

According to Lazarov (2007), the most frequent allergens triggering allergic contact dermatitis are 2-hydroxyethyl methacrylate (2-HEMA) and 2-hydoxypropyl methacrylate (2-HPMA). These common sensitizers may produce a cross-reaction with other acrylic compounds and may trigger allergic reactions when re-exposure occurs in a different setting.

Prior to polymerization, compounds of denture base resins can cause hypersensitization and allergy to dental laboratory staff and, after polymerization, to the denture wearer due to the continued release of residual monomers (Lassila & Vallittu, 2001).

If allergy is suspected, investigation for immediate or delayed type of hypersensitivity is indicated, using patch testing, prick testing and blood testing for allergen-specific IgE (Gawkrodger, 2005).



In a clinical case report by Koutis & Freeman (2001), patch testing showed allergic reactions to samples of the denture material and to 2-hydroxyethyl methacrylate. The prolonged boiling of the denture resulted in a reversal of the patient symptoms and subsequent samples of these fully-cured denture material produced negative patch tests.

The information in the previous paragraphs is important to make clinicians aware of the biological effects of materials and the consequences of different polymerization methods. Empowering clinicians to influence their laboratory technicians in selecting materials with minimal cytotoxicity (Jorge *et al.,* 2003), and to adapt clinical practice to minimize exposure.

### 1.4.11. Biocompatibility

According to Polyzois (1994) biocompatibility can be described as *"The ability of a material to perform with an appropriate host response in a specific application. Appropriate host response means no (or a tolerable) adverse reaction of a living system to the presence of such a material. An adverse reaction may be due to the toxicity of a dental material. Therefore toxicity may be regarded as one reason for non biocompatibility of a dental material. The toxicity of a dental material can be evaluated by in vitro tests, animal experiments and clinical trials. There exists a variety of different in vitro test methods."*

To evaluate possible reactions to acrylic resin, Kaaber (1990) suggested that factors such as oral diseases, systemic disorders unrelated to the prosthesis, and other common causes such as trauma, poorly-adjusted dentures, and chemical injury should be taken into consideration.



### 1.4.12. Cytotoxicity of acrylic resins

Although acrylic resins are widely used for dentures as well as for relining and repairing dentures, no biologic testing is required for their use in dentistry, because they are considered to be low-risk for patients' health (Lefebvre *et al.*, 1994). Although occupational hazards such as chronic gastritis and dermatological reactions have been reported (Vallittu, 1996), the material is regarded as safe for patients in the sense that there are no indications that the release of MMA from dentures causes systemic effects in patients (Phillips, 1991).

However, substances leaching from acrylic resin denture bases have repeatedly been shown to be cytotoxic by *in vitro* testing. Although residual monomer and formaldehyde are most commonly referred to in terms of the cytotoxicity of denture resins, other potentially toxic substances such as benzoic acid, dibutyl phthalate, phenyl benzoate, phenyl salicylate and dicyclohexyl phthalate have also been found to eluate from denture resins (Lefebvre *et al.*, 1991; Tsuchiya *et al*., 1994; Lygre *et al.*, 1995; Jorge *et al.*, 2003 ) .

In 1991, Lefebvre *et al*. showed that denture base resins had an effect on oral epithelial cells using an *in vitro* epithelial cell culture system. The researchers used three light-polymerized resins, but the cytotoxicity appeared to be related to the formulation of the material and not to the type of polymerization. Varying the polymerization time or changing the light-polymerization unit appeared to have little effect on the results.



In a later study, the same authors tested the cytotoxicity of four light- and one heat-polymerized denture resin. Samples of the eluates of light- and heat-polymerized denture- base resins inhibited cell metabolism directly after transfer. Aged eluates (after 30 days of storage) stimulated and then inhibited the responses, suggesting that the components that leach out of the tested materials do so at different rates and have a prolonged toxic effect on cells (Lefebvre *et al.*, 1994).

Schuster *et al*. (1995) explained the *in vitro* cytotoxic effect by the fact that the metabolism of several lipid classes, found in the cell membrane, is altered by

several resin eluates. They report that this alteration of the cell lipids and the presence of the previously unrecognized lipids, may be the reason for some clinical evidence of cytotoxicity and allergic reactions.

Sheridan *et al.* (1997), using human gingival fibroblasts and MTS assay, found that eluates from microwaved, heat cured and chemically cured resin disks were cytotoxic to human fibroblasts at all time periods tested (up to 96 hours). Interestingly enough, viability was less impacted as disk immersion time increased. Eluates from chemically-activated resin disks were more cytotoxic than eluates from heat-activated and microwave-activated disks. These researchers also reported that the cytotoxicity appeared to diminish as disk immersion time was increased. The greatest cytotoxic effect on cell viability was observed in their study with eluates recovered after 24 hours of disk immersion, and the least cytotoxic effect was reported in eluates recovered after 96 hours of immersion.



Kedjarune *et al.* (1999), using human oral fibroblasts and the MTT assay at 24 and 48 hours after processing, found that MMA tested in the same concentrations as the MMA found leached from acrylic resin in saliva, was toxic to *in vitro* cell cultures.

Cimpan *et al.* (2000) demonstrated cytotoxicity using human monoblastoid cells, by inducing cell death by apoptosis and necrosis. According to Cimpan *et al.* (2000), disks and eluates of all of the tested polymers enhanced cell death by apoptosis and necrosis. They found that the toxic effects were stronger in the case of direct contact of the cells on the polymer disks than when eluates were used. Three of the four auto-polymerized polymers yielded higher percentages of

apoptosis and necrosis than the heat-polymerized polymers. The results of their study indicated that eluates from PMMA denture base polymers induced cell death.

Huang *et al.* (2001) also reported on the cytotoxicity of denture base resins using human buccal fibroblast cultures. They concluded that the cytotoxicity results depended on the materials tested and the cell culture system used.

Using MTT and $^{3}$H-thymidine incorporation assay, Jorge *et al*. (2004) examined the effect of post-polymerization heat treatment on the cytotoxicity of three denture- base resins (heat-, rapid-, and microwave-polymerized). The post-polymerization heat treatments were warm water (55ºC for 60 minutes) and microwaving (500 W for 3 minutes). They found that all three resins, regardless of the post-polymerisation treatment, were slightly cytotoxic to L929 cells using the $^{3}$H-thymidine incorporation assay. In contrast, with the MTT assay, the eluates from all resins were categorized as non-cytotoxic even though there was a slight reduction in the cytotoxicity of the resins when treated to warm water after polymerization. This study showed that the $^{3}$H-thymidine incorporation assay was more sensitive than the MTT assay in detecting resin cytotoxicity. Because post-polymerization treatment, such as warm water soaking, which is expected to reduce residual monomer content and monomer leaking, did not reduce the cytotoxicity of the resins using the $^{3}$H-thymidine assay, it was speculated that other cytotoxic substances, besides monomer, continued to leak from the resin.

In 2007, the same authors tested post-polymerization treatments and different cycles of polymerization on the cytotoxicity of denture base resins using the $^{3}$H-thymidine incorporation assay. Each of these resins was exposed to short and long polymerization cycles. Surprisingly, the researchers found that a longer polymerization cycle increased the cytotoxicity of one of the tested heat-cured denture bases. After exposing the latter to warm-water post-polymerisation, its cytotoxicity was subsequently reduced (Jorge *et al*., 2007).

### 1.4.13. *In vitro* cytotoxicity testing

Human studies are ethically prohibitive and animal studies have become increasingly controversial. In addition, both are often associated with uncontrollable variables. On the other hand, testing biomaterials using cell cultures is relatively easy, reproducible and controllable (Jorge *et al*., 2003).



However, it is important to bear in mind that all assays oversimplify the events that they measure and are used because they are cheap, easily quantified, and reproducible (Freshney, 1994:287).

"*The most widely used biological systems for toxicity screening of dental materials are cell cultures. Cell cultures for toxicity screening of dental materials are valuable tools for understanding their biological behaviour, even if the limitations of the methods are taken into consideration, especially concerning the interpretation of the results."* (Freshney, 1994:287).

### 1.4.13.1. Cell viability

"*Once a cell is explanted from its normal in vivo environment, the question of viability, particularly in the course of experimental manipulation, becomes fundamental. The data is not acceptable unless the greater majority of the cells are shown to be viable.*

*Aspects influencing the growth or survival: growth is generally taken to be regenerative, measured by clonal growth, net change in population size, as in a growth curve or, change in cell mass, or gross metabolic activities such as respiration or DNA, RNA or protein synthesis*." (Freshney, 1994:287).

### 1.4.13.2. The nature of the assay

"*The choice of assay will depend on the agent under investigation, the nature of response and the particular target cell. Assays can be divided into 2 major classes: (1) an immediate or short term response such as alteration in membrane permeability or perturbation of a particular metabolic pathway, and (2) long term survival, either absolute, usually measured by the retention of self-renewal capacity.*" (Freshney, 1994:149).



### 1.4.13.3. The MTT assay test

According to A. Doyle and J.B. Griffiths in their textbook entitled "Cell and Tissue Culture: Laboratory Procedures in Biotechnology", 1998, they describe the MTT assay as follows:

"*The MTT assay (Mosmann, 1983) is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells.*

*The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water soluble substrate 3-(4,5-dimethylthylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue Formozan product that is soluble in water. The amount of Formozan produced is directly proportional to the cell number in a range of cell lines (Mosmann 1983; Gerlier & Thomasset., 1986; Grailer et al., 1988; Al-Rubeai & Spier., 1989). The results are consistent with those obtained from $^{3}$H-thymidine uptake assays. The MTT assay is more useful in the detection of cells that are not dividing but still active. It can, therefore, be used to distinguish between proliferation and cell activation (Gerlier & Thomasset., 1986). The technique permits the processing of a large number of samples with a high degree of precision using a multiwell scanning spectrophotometer (micro- ELISA reader)."*



The most commonly used MTT assay, also used in this experiment, is the suspension, or monolayer cells procedure. An alternative procedure is the MTT assay – immobilized cells as described by Al- Rubeai *et al. (*1990).

The $^{3}$H-thymidine incorporation, which reflects DNA synthesis levels, may be used to support MTT tests. As mentioned previously in the literature, Jorge *et al.* (2004) stated that $^{3}$H-thymidine incorporation proved to be more sensitive for cytotoxicity testing.

# CHAPTER 2

# METHODOLOGY

## 2.1. Aim

The aims of this *in vitro* study were:

a) To perform a controlled quantitative analysis of the influence of pre- and post-polymerization procedures on the sorption and solubility of a heat-polymerized denture base resin, and

b) To assess the cytotoxicity of the same heat-polymerized resin subjected to selected post-polymerization treatments.



## 2.2. Objectives

The objectives were as follows:

1. To determine if *polishing* the surface of a heat-polymerized denture base resin changes its sorption and solubility properties.

2. To determine if altering the *mixing ratios* of a heat-polymerized denture base resin changes its sorption and solubility properties.

3. To determine if different *temperatures* of water immersion after processing a heat-polymerized denture base resin would change its sorption and solubility properties.

4. To determine if different *thicknesses* of a heat-polymerized denture base resin have an effect on its sorption and solubility properties.

5. To determine if different *temperatures* of water immersion after processing a heat-polymerized denture base resin have an influence on its flexural strength.

6. To support the solubility findings with *in vitro* cytotoxicity tests, by using the same denture base resin and subjecting it to those post-polymerization treatments that have been shown to reduce its solubility.

**2.3. Null hypotheses**

1. There is no difference in water sorption and solubility between an *unpolished*, and *polished,* denture base polymer.

2. There is no difference in water sorption and solubility among different *mixing ratios* of a denture base polymer.

3. There is no difference in water sorption and solubility among the different *water immersion temperatures* of a denture base polymer.



4. There is no difference in the water sorption and solubility of a denture base polymer with a variance in *thickness*.

5. There is no change in the *flexural strength* after a denture base polymer has been submerged in water at different temperatures.

6. There is no difference in the *cytotoxicity* among eluates from untreated specimens, polished specimens and specimens that have been submerged in warm water.

## 2.4. Methods and materials

### 2.4.1. Introduction

The International Standard Organization (ISO) 1567:1999(E) standard for sorption and solubility testing was used as a basis for the experiments. The conditions as prescribed by the ISO standard were followed as strictly as possible. Some deviations were necessary for the purpose of a particular test. These deviations will be addressed appropriately in the methodology and discussion sections.

In addition to the ISO standard, manufacturers' recommendations in terms of material handling were adhered to, except when the test conditions required a deviation. These deviations will again be addressed appropriately in the relevant sections of this paper.



For the manufacturing of the specimens for the first experiment (polishing), disk replicas were cut from 2 mm thick mouth-guard silicone sheets (Proform™ mouth guards, Dental Resources Inc., Delano, MN). For the second (ratios) and third (water-immersion temperatures) experiment, disk replicas were made from solid metal sheets of 1 mm and 0.5 mm, respectively, by an instrument maker. For the first two experiments, molds were made from the disk replicas, using yellow stone (Heraeus Kulzer Inc., NY, USA) and brass flasks (R-020080 Round flask, Mestra, Bilboa, Spain). Due to the thinness of the 0.5 mm disk replicas of the third experiment, the disk replicas were invested in Die-Keen® Resin reinforced (Heraeus Kulzer Inc., NY, USA) instead of yellow stone for additional precision.

For the cytotoxicity tests, disk replicas were made from dental modelling wax (Associated Dental Products Limited, Wiltshire. UK) and invested in yellow stone (Heraeus Kulzer Inc., NY, USA) and brass flasks (R-020080 Round flask, Mestra, Bilboa, Spain).

For each specimen, a separate mix was made as specified by the ISO. The powder was accurately weighed off for each specimen, using an analytical scale with a 0.01 gram precision (T5400D, OHAUS corporation, NJ, USA).

For each experiment, disks were numbered with a black waterproof marker and randomly assigned to a test group.

Data were captured, using software package MSExcel.



### 2.4.2. Sorption and solubility testing

For all the sorption and solubility tests, disk-shaped specimens from a Type 1, Class 1 (ISO classification) denture base polymer (Vertex, Rapid Simplified, Vertex-Dental B.V, Zeist, NL), were prepared.

Immediately after manufacturing, the disks were placed on custom-made drying racks, keeping the specimens parallel to each other and separated. The racks with the disks were placed in a desiccator with silica gel, freshly dried beforehand, for 300 minutes, at 130°C. The desiccator containing the specimens was incubated at 37°C for 23±1 hours. After this time, the racks with disks were removed from the desiccator and placed into a second desiccator, again, with freshly-dried silica gel. The second desiccator was kept at room temperature. After 60 minutes in the

second desiccator, the specimens were ready for weighing, one by one, using a digital analytical scale (Mettler AE240) with an accuracy of 0.0001 mg. After all the disks had been weighed, the racks with disks were returned to the first desiccator, and placed into the oven at 37ºC for 23±1 hours, until the next weighing process which was a repetition of the process described above.

This drying and weighing protocol was repeated until the loss in mass of each specimen was not more than 0.2 mg between two successive weighing procedures. This conditioned mass was called M1. At this stage, the volume (V) for each disk was calculated by using the mean of 3 diameter and 5 thickness measurements. After determining M1 and V, the racks with disks were submerged in distilled water. After incubation at 37°C for 7 days, the disks were removed from the water, wiped with a clean, dry towel, waved in the air for 15 seconds and weighed one by one. This mass was recorded as M2. The drying protocol as explained for M1 was repeated. The constant mass that was reached after the second drying and weighing routine was recorded as M3. The disks were always handled with polymer-coated tweezers.



Water sorption (Wsp) in µg/mm³ was calculated using the following formula:

$$W_{sp} = \frac{M_2 - M_3}{V}$$

Water solubility (Wsl) in µg/mm³ was calculated using the following formula:

$$W_{sl} = \frac{M_1 - M_3}{V}$$

### 2.4.3. Experimental groups

#### 2.4.3.1. Polishing group

Twenty four specimens, 50 mm in diameter and 2 mm thick, were prepared (Figure 2). To reduce the risk of fracturing during polishing, the specimen thickness of 0,5 mm, as recommended by ISO, was increased to 2 mm. The prepared specimens were numbered and randomly divided into 2 groups of 12 specimens each. The test specimens (n = 12) were polished, following ISO polishing procedures, while the control specimens (n = 12) remained unpolished. The Wsp and Wsl values of the 2 groups were compared using the Mixed procedure.

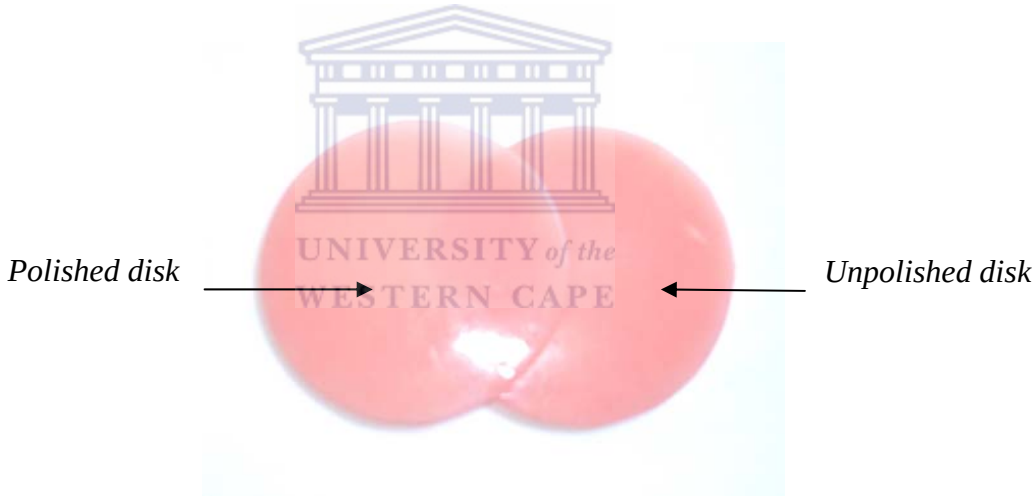


*Figure 2: Example of a polished and unpolished disk*

#### 2.4.3.2. Mixing ratios

Thirty six specimens, 50 mm in diameter and 1 mm thick, were prepared. The manufacturers' instructions and procedures were followed, except for the mixing ratios of the test groups. The specimens of the control group were made using the recommended ratio, while the 2 test groups (n = 12) had 25% more and 20% less powder by weight than the control group. The percentage of altered powder ratios

were determined by a pilot study. The altered ratios represented mixes that allowed handling of the dough, as described by the manufacturer. The Wsp and Wsl values of the 3 groups were compared, using the Mixed procedure.

Instead of the 0.5 mm thickness as specified by ISO, 1 mm thick specimens were prepared. The 1 mm control group specimens will also be used to compare the influence of different thicknesses (0.5, 1 and 2 mm) on sorption and solubility, as will be described in paragraph 2.4.3.4.

#### 2.4.3.3. Water-immersion temperature

Fifty six specimens, 50 mm in diameter and 0.5 mm thick, were randomly divided into 4 groups of 14 specimens each, after M1 had been established. Each group was immersed in distilled water at different temperatures (22°C, 37°C, 55°C and 70°C) during the 7-day water-immersion stage of the sorption and solubility testing. The Wsp and Wsl results of the 4 groups were compared, using a non-parametric analysis, the Median test.



#### 2.4.3.4. Thickness

The Wsp and Wsl results of the control groups of the 3 previous tests (polishing – 2 mm; mixing ratios – 1 mm; and water immersion temperature – 0.5 mm) were compared. The groups were compared by pairwise comparison and the Median test.

### 2.4.3.5. Flexural strength

Specimens of the 4 groups used for the water temperature testing in 2.4.3.3. were used. The disks were modified into strips of 10 mm wide, ensuring not to overheat the specimen during grinding. Using a digital calliper (Mitutoyo America Corporation, U.S.A.) with a 0.01 mm resolution, 3 measurements were made of the height and thickness. The average of the 3 measurements was used in the equation to calculate the flexural strength. The 56 strips were immersed into distilled water at 37±1 °C for 50±2 hours prior to flexural testing. Each specimen strip was removed from the water immediately before being positioned onto the supports of the flexural test rig in a universal testing machine (Zwick Roell, Germany). A load was applied by a centrally positioned rod with a round tip. A constant crosshead speed of 5 mm/minute was maintained until the specimen fractured. The force at break in N was used to calculate the flexural strength (Ó), in megapascal, using the following equation:



$$Ó = 3Fl / 2bh^2$$

with

***F*** being the maximum load in N exerted on the specimen

***l*** being the distance in mm between the supports

***b*** being the width in mm of the specimen measured before water immersion

***h*** being the height in mm of the specimen measured before water immersion.

The flexural strength of the 4 different temperature groups was compared using the Median test.

#### 2.4.3.6. Cytotoxicity testing

To investigate the potential cytotoxicity of the eluate from the denture-base resin, an *in vitro* method previously used for denture cytotoxicity testing by Campanha *et al.* (2006) was adapted and combined with the standard MTT method for cytotoxicity testing. A pilot study was conducted to expose any problem areas and to be of value for the possible cytotoxicity over time. Thereafter, the final experiment was executed.

##### 2.4.3.6.1. Fabrication of specimens

For the pilot study, 12 disks (10 x 1 mm) were prepared from the same denture base resin, under aseptic conditions, and following the manufacturers' instructions. After polymerization the specimens were treated as follows:

Group 1: specimens (n = 4) were not submitted to any treatment after polymerization (untreated group).



Group 2: specimens (n = 4) were polished after polymerization (polished group).

Group 3: specimens (n = 4) were submerged in distilled water, at 70ºC, for 7 days, after polymerization (temperature group).

The averages of 3 readings from each of the 4 disks were used as an observation in data analysis. This brought the number of observations in the raw data to four values per group.

For the final test, 27 disks (10 x 1 mm) of the same denture base material were prepared, under the same conditions as the pilot study. The same 3 test groups had

9 specimens each. One disk accounted for one observation in this test by means of triplicate density readings per specimen.

The Means procedure was used in data analysis.

For decontamination purposes, all the disks were exposed to ultraviolet light, in a sterile laminar-flow cabinet, at room temperature, for 20 minutes, prior to testing.

**2.4.3.6.2. Eluate preparation**

Each disk was placed in the well of a sterile, 12-well plate (well diameter ± 22/23 mm), adding 3 ml of Dulbecco modified eagles medium (DMEM), supplemented with 10% bovine serum and 1% penicillin/ streptomycin (Cambrex Bio Science, Highveld Biological, SA) (Figure 3). A standard eluate preparation was followed and distributed into the different wells, with and without disks. Medium without disks was also incubated in another 12-well plate to serve as a negative control. Therefore, in figure 3, four groups of eluates are observed (number 1 - 4) in the 12-well plates, with four wells per group (A-D) for each disk.

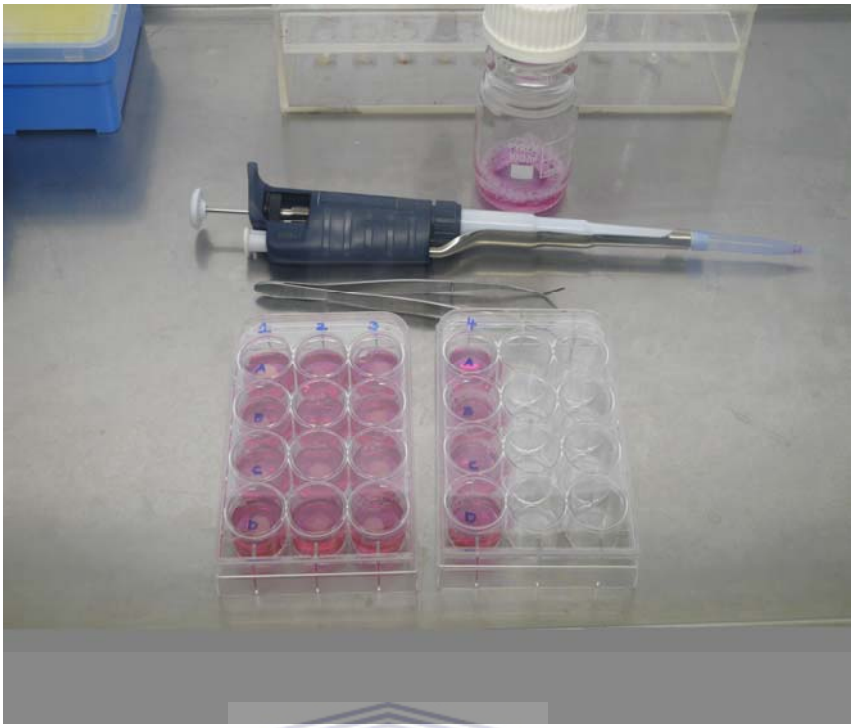

*Figure 3: Specimens in 12-well plates for eluate preparation.*



**2.4.3.6.3. Cell culture methods**

Established Balb/c 3T3 mouse fibroblast cells were obtained from the National Repository for Biological Materials (Sandringham, South Africa.). Cells were incubated under standard conditions (37ºC, under 5% carbon dioxide and 95% humidity) in DMEM supplemented with 10% bovine serum and 1% penicillin/ streptomycin (Cambrex Bio Science Highveld Biological, SA). Cells were sub-cultured, using trypsin/EDTA, and only cells from passage 3 - 6 were used. Cells were grown to near confluence (strong growth phase), then trypsinized and plated out onto 96-well plates with 100ml medium and allowed to attach for 48 hours. Cells were left undisturbed and only removed from the incubator once, to check for contamination, by microscope.

#### 2.4.3.6.4. Cytotoxicity test

Three 100 µl aliquots of the eluate from each disk were transferred to 3 wells (3 wells with 100µl in each) of a 96-well plate containing the fibroblasts, replacing the original medium of 100µl. After being left, undisturbed, for 24 hours in the incubator, the MTT test was performed. Wells with medium only, from the control 12-well plate, served as control.

After the eluate had been in contact with the cells for 24 hours (in the pilot study) 10 µl of MTT (5 mg/ml) was added to the 100 µl of medium in each well and incubated for 3 hours. The cultures were removed from the incubator, and the resulting formazan crystals were dissolved by adding 100 ml of DMSO (Sigma Chemical). The plates were shaken at low speed, for a short time, until the crystals were completely dissolved. The absorbance was spectrophotometrically measured on a Kayto RT 2100C micro-plate reader at a wavelength of 540 nm to determine the number of viable cells. This process was repeated after the eluate had been in contact with the cells for 1, 2, 3 and 4 weeks, respectively.



In the second MTT testing, the same procedures as described above were followed and the cells were tested after 24 and 48 hours of contact time with the eluates.

The varying results wil be described in Chaper 3.

## 2.5. Statistical analysis

For each experiment, descriptive and analytical statistics of the results will be presented. Visual presentation by means of box and whisker plots or scatter plots will be provided.

The differences were identified at a significance level of 0.01. The 0.01 probability level was chosen due to the greater significance factor.

The software SAS v9 (SAS Institute Inc., Cary, NC, USA) was used for the analysis.

# CHAPTER 3

# RESULTS

### 3.1. Influence of polishing on sorption and solubility

The first disk of the 24 specimens, reached M1 on day 8, while the last disk reached M1 on day 15 of the conditioning process. M2 was reached after 7 days of immersion in water. The first disk reached M3 on day 28 of the second conditioning protocol, while the last specimens reached M3 on day 32 (Table I).



| Test | Conditioning stage | First disk to reach stable weight (days) | Last disk to reach stable weight (days) | Total time required to complete test (days) |
|---|---|---|---|---|
| Polishing | M1 | 8 | 15 | 54 |
| | M2 | 7 | | |
| | M3 | 28 | 32 | |
| Ratio | M1 | 7 | 15 | 64 |
| | M2 | 7 | | |
| | M3 | 38 | 42 | |
| Temperature | M1 | 5 | 7 | 34 |
| | M2 | 7 | | |
| | M3 | 18 | 20 | |

*Table I: Summary of the time required to complete the 3 sorption and solubility experiments.*

Wsp and Wsl per unit volume were calculated for each specimen.

Three specimens in the polished group did not reach a stable mass during the conditioning process and were not used in the analysis of the results. The descriptive statistics are shown in table II.

| | Wsp | | Wsl | |
|---|---|---|---|---|
| | *Polished* | *Unpolished* | *Polished* | *Unpolished* |
| Maximum | 24 | 24 | 1.1 | 1.9 |
| Minimum | 21 | 22 | 0.3 | 0.6 |
| Median | 22 | 23 | 0.6 | 1.0 |
| Mean | 22 | 23 | 0.6 | 1.1 |
| St dev | 0.882 | 0.651 | 0.268 | 0.371 |
| N | 9 | 12 | 9 | 12 |

*Table II. Descriptive statistics of the water sorption and solubility in µg/mm³ of the polished and unpolished groups. Wsp = water sorption, Wsl = water solubility.*



The mean values of Wsp and Wsl of the polished and unpolished groups were compared, using the Mixed procedure. For Wsp, there was no significant difference between the 2 groups ($p = 0.53$). However, for Wsl, there was a significant difference between the groups ($p = 0.0056$). The mean of the unpolished group is about 0.45 units higher than that of the polished group, using the 0.01 significance level; the 95 confidence interval estimated difference is 0.15 and 0.75, respectively, for Wsp and Wsl. Figures 4 and 5 show the box plots for Wsp and Wsl of both groups.

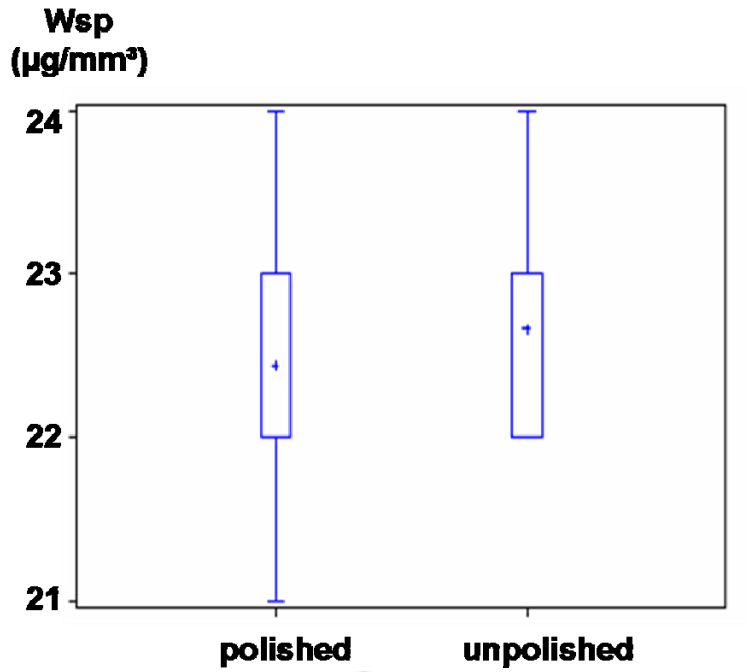


*Figure 4. Box and whiskers plot for the water sorption in µg/mm³ of the polished and unpolished groups. Wsp = water sorption.*



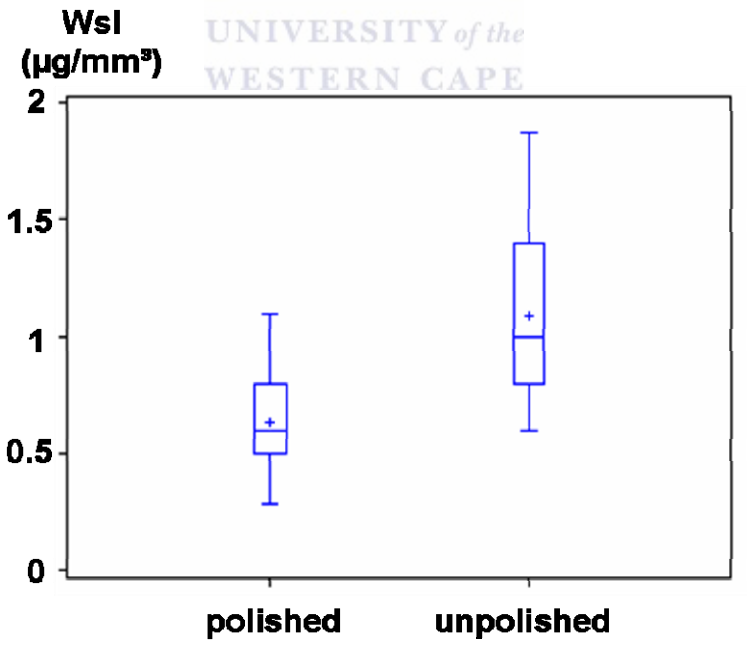


*Figure 5. Box and whiskers plot for the water solubility in µg/mm³ of the polished and unpolished groups. Wsl = water solubility.*

### 3.2. Influence of mixing ratios on sorption and solubility

The first specimen, from a total of 36 specimens, reached M1 on day 7. The last specimen reached M1 on day 15. After immersion in water for 7 days, M2 was established. Following a second conditioning process, M3 was reached on day 38 by the first specimen and on day 42 by the last specimen (Table I).

Wsp and Wsl per unit volume were calculated for each specimen. The descriptive statistics for the 3 groups are shown in tables III and IV.

| **Wsp** | | | |
|---|---|---|---|
| | *Control* | *Liquid* | *Powder* |
| Maximum | 27 | 27 | 40 |
| Minimum | 21 | 22 | 21 |
| Median | 25 | 25 | 23 |
| Mean | 24 | 25 | 27 |
| Std Dev | 2.172 | 1.187 | 7.796 |
| N obs | 9 | 12 | 8 |



*Table III. Descriptive statistics of water sorption in µg/mm³ of the 3 ratio groups. Liquid = the group with a higher liquid content. Powder = the group with a higher powder content. Wsp = water sorption.*

| Wsl | | | |
|---|---|---|---|
| | *Control* | *Liquid* | *Powder* |
| Maximum | 7.1 | 5.9 | 2.4 |
| Minimum | 0.6 | 0.9 | 0.4 |
| Median | 1.6 | 2.1 | 1.5 |
| Mean | 2.5 | 2.3 | 1.4 |
| Std Dev | 2.178 | 1.326 | 0.615 |
| N obs | 9 | 12 | 8 |

*Table IV. Descriptive statistics of water sorption in µg/mm³ of the 3 ratio groups. Liquid = the group with a higher liquid content. Powder = the group with a higher powder content. Wsl = water solubility.*

The number of observations is different for the 3 groups. Originally, 12 specimens per group were manufactured and subjected to testing. However, some specimens from the control and powder group were not used for data analysis, because they had not reached a constant mass.



The three ratio groups were compared using the Mixed procedure. There were no significant differences for Wsp ($p = 0.34$). For Wsl, there were small but insignificant differences ($p = 0.0683$). Looking at the medians of the groups, the pair of groups that differs the most (but not significantly) is the liquid and powder group. This may not look like the 'closest' pair when considering their mean values, but when considering how close they are relative to the standard deviations this does qualify as the "closest" pair.

The liquid group has a smaller standard deviation than the control group, hence, in relative terms (difference in means divided by the standard deviation of the difference), the liquid group is 'further' from powder than the control group.

Figures 6 and 7 show the box plots for the Wsp and Wsl results in the 3 ratio groups.

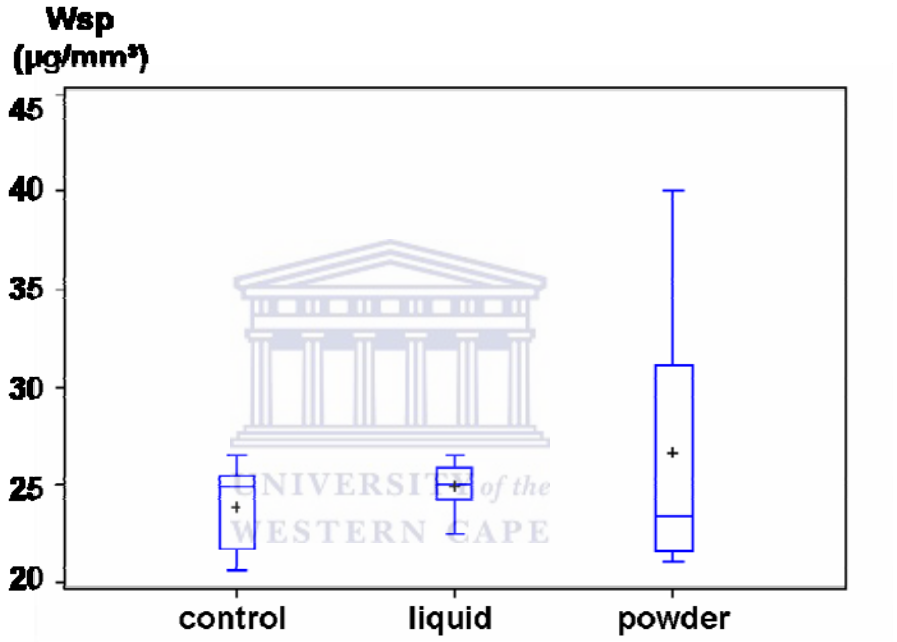


*Figure 6. Box and whiskers plot for the water sorption in µg/mm³ of the 3 ratio groups. Wsp = water sorption. Liquid = the group with the higher liquid content. Powder = the group with the higher powder content.*

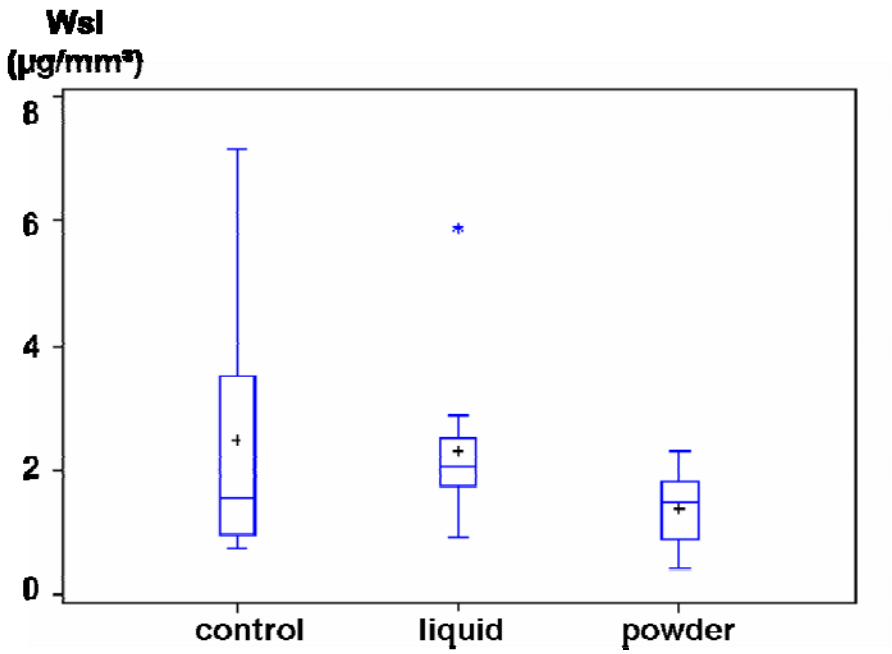


*Figure 7. Box and whiskers plot for the water solubility in µg/mm³ of the 3 ratio groups. Wsl = water solubility. Liquid = the group with the higher liquid content. Powder = the group with the higher powder content.*

One outlier can be observed in the Wsl box plot of the liquid group. This specified specimen also gained in mass after water immersion, like all the other specimens, but lost more mass during the conditioning process, after water immersion, compared to the other specimens in the liquid group. The reason for this outcome is not known. This specimen was not excluded from the results as it had been treated according to the same standardized procedure as the other specimens in its group.

### 3.3. Influence of temperature on sorption and solubility

The first of the 56 disks reached M1 on day 5, while the last disk reached M1 on day 7. The disks were immersed into water for 7 days after which M2 was established. M3 was first reached on day 19 with the last specimens of the group reaching M3 on day 20 (Table I). The 4 groups of 14 specimens each were compared using non-parametric analysis, i.e. the Median test. Table V and VI show the descriptive statistics.

| **Wsp** | | | | |
|---|---|---|---|---|
| | *22ºC* | *37°C control* | *55ºC* | *70°C* |
| Maximum | 39 | 39 | 33 | 29 |
| Minimum | 31 | 30 | 28 | 27 |
| Median | 34 | 34 | 30 | 28 |
| Mean | 34 | 33 | 30 | 28 |
| Std Dev | 2.129 | 2.437 | 1.562 | 0.632 |
| N obs | 14 | 14 | 14 | 11 |

*Table V. Descriptive statistics of water sorption in µg/mm³ for the 4 temperature groups. Wsp = water sorption*

| **Wsl** | | | | |
|---|---|---|---|---|
| | *22ºC* | *37°C control* | *55ºC* | *70°C* |
| Maximum | 0.2 | 1.1 | 2.4 | 3.7 |
| Minimum | 0.0 | 0.1 | 0.3 | 0.4 |
| Median | 0.0 | 0.6 | 0.7 | 1.5 |
| Mean | 0.0 | 0.6 | 0.9 | 1.5 |
| Std Dev | 0.065 | 0.268 | 0.514 | 0.885 |
| N obs | 14 | 14 | 14 | 12 |

*Table VI. Descriptive statistics of water solubility in µg/mm³ for the 4 temperature groups. Wsl = water solubility.*

The 70°C group only contained 12 specimens compared to the other groups with 14 specimens. In the 70°C group, one specimen fractured during preparation and two specimens were not used for data analysis due to the same reason as described in paragraph 3.2.

Figures 8 and 9 show the box plots of the sorption and solubility for the 4 temperature groups.

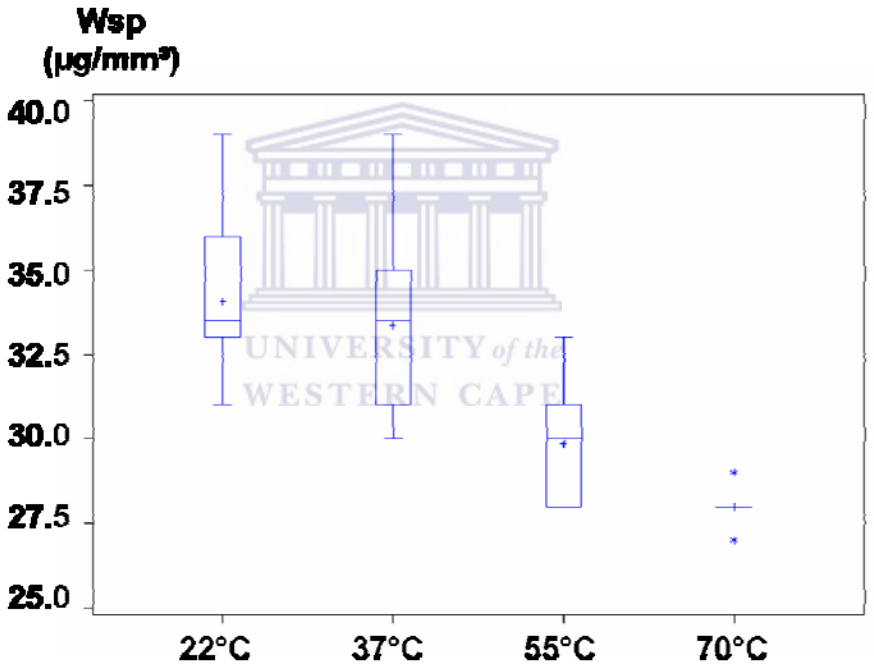


*Figure 8. Box and whiskers plot for the water sorption in µg/mm³ of the 4 temperature groups. Wsp = water sorption.*

For Wsp, indications of 2 outliers can be observed in the 70°C group. Observing the data of the 70°C group, indicated the outliers as the maximum and minimum value specimens. This result does not deviate much from the mean value of the

70°C group if it is compared to the other groups' mean value in relation to the maximum and minimum values. Due to the close grouping of the 70°C test group, the maximum and minimum value specimens present as outliers even though this was not the case if the results were observed in the context of the entire test.

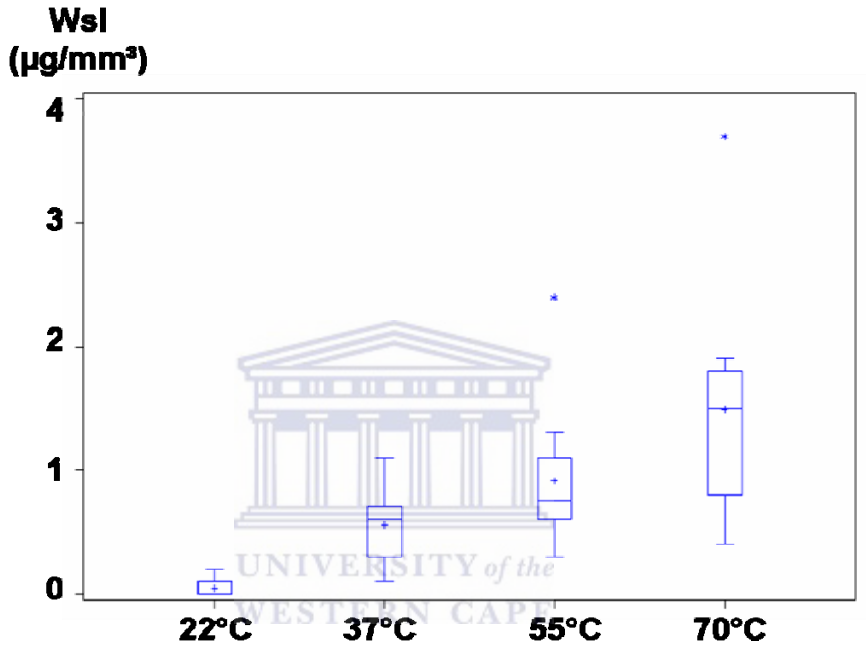


*Figure 9. Box and whiskers plot for the water solubility in µg/mm³ of the 4 temperature groups. Wsl = water solubility.*

For Wsl, an outlier in the 55 °C and 70°C group was found. Both values are extremely high compared to the rest of each groups spread. The outliers lost more in mass during the second drying-out process after water immersion, than their original mass before water immersion. The specimens had been treated to the same processing and treatment conditions in the specific groups and no reason for this behaviour difference in solubility could be found. Therefore, the specimens were not excluded from the data.

These extreme values indicate non-normal data and different variability across the temperature groups. With these facts in mind, a formal statistical test, comparing the temperature groups, should be non-parametric. All four groups were compared using the Median test. Since a highly significant difference was found ($p<0.0001$), the 6 possible pairwise comparisons were performed. Again, the Median test was used for these comparisons. In view of the number of comparisons being done, a more stringent level of 0.01 (rather than the usual 0.05) was selected for significant differences between pairs. For Wsl, all pairs were significantly different at the 0.01 level, except the 37ºC group, compared to the 55ºC group and the 55ºC group, compared to the 70ºC group. For Wsp, all pairs were significantly different at the 0.01 level, except the 22 º C group, compared to the 37ºC group.



**3.4. Influence of thickness on sorption and solubility**

To determine the influence of thickness, the Wsp and Wsl of the control groups of the three previous tests (unpolished with specimens of 2 mm thickness, mixing ratios with specimens of 1 mm thickness, and, temperature with specimens of 0.5 mm thickness) were compared by means of the Median test and pairwise comparison.

The descriptive statistics for each thickness group are shown in tables VII and VIII.

| Wsp | | | |
|---|---|---|---|
| | *Group1 (0.5mm)* | *Group2 (1mm)* | *Group3 (2mm)* |
| Maximum | 39 | 27 | 24 |
| Minimum | 30 | 20 | 22 |
| Median | 34 | 25 | 23 |
| Mean | 33 | 24 | 23 |
| Std Dev | 2.437 | 2.163 | 0.651 |
| N obs | 14 | 9 | 12 |

*Table VII. Descriptive statistics of water sorption in µg/mm³ for the 3 thickness groups. Wsp = water sorption*



| Wsl | | | |
|---|---|---|---|
| | *Group1 (0.5mm)* | *Group2 (1mm)* | *Group3 (2mm)* |
| Maximum | 1.1 | 3.5 | 1.9 |
| Minimum | 0.1 | 0.8 | 0.6 |
| Median | 0.6 | 1.2 | 1.1 |
| Mean | 0.6 | 1.5 | 1.1 |
| Std Dev | 0.268 | 0.950 | 0.377 |
| N obs | 14 | 9 | 12 |

*Table VIII. Descriptive statistics of water solubility in µg/mm³ for the 3 thickness groups.Wsl = water solubility*

In group 1 (0.5 mm thickness) there were fourteen specimens, group 2 (1mm thickness) only had nine of the original twelve specimens, and group 3 (2mm group) had twelve specimens.

The two box plots and a scatter plot demonstrate the distribution of the 3 groups (figures 10, 11 and 12). The grouping does blur some potentially interesting relationships due to the variance in thickness between the groups. Using the Median tests to compare the mean responses for the three groups, it was found that Wsl ($p=0.0051$) and Wsp ($p<0.0001$) differed significantly among the 3 groups. Pairwise comparisons show significant differences between the 0.5 mm group and each of the 1 mm and 2 mm groups respectively, but the 1 mm and 2 mm groups did not differ from each other at the 0.01 level.

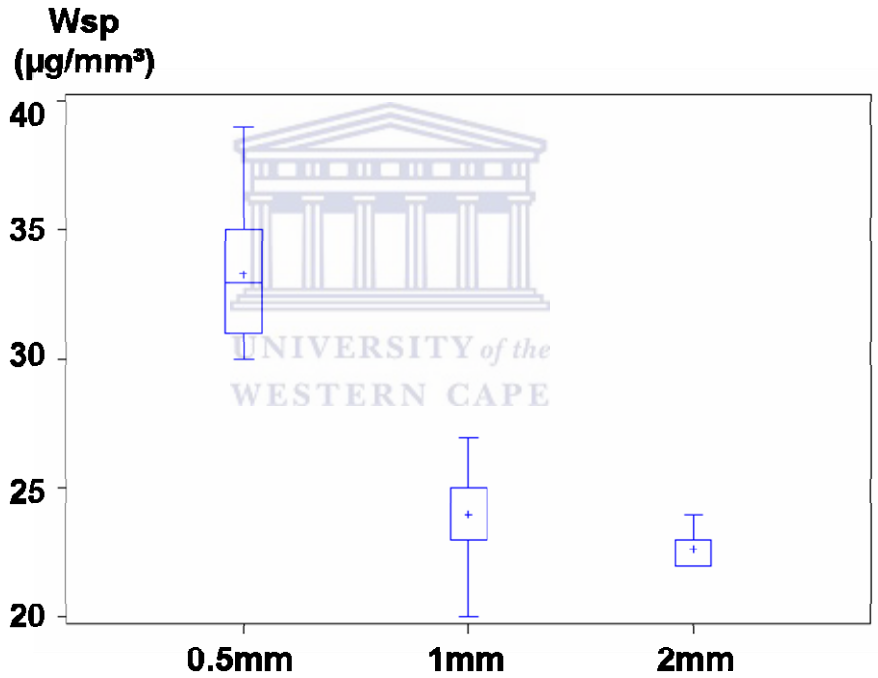


*Figure 10. Box and whiskers plot for the water sorption in µg/mm³ of the 3 thickness groups. Wsp = water sorption.*

In observing the box and whisker plots for Wsp (figure 10), the 1 mm and 2 mm groups were the closest pair. Much higher levels of Wsp can be observed in the 0.5 mm group, when compared to the other 2 groups.

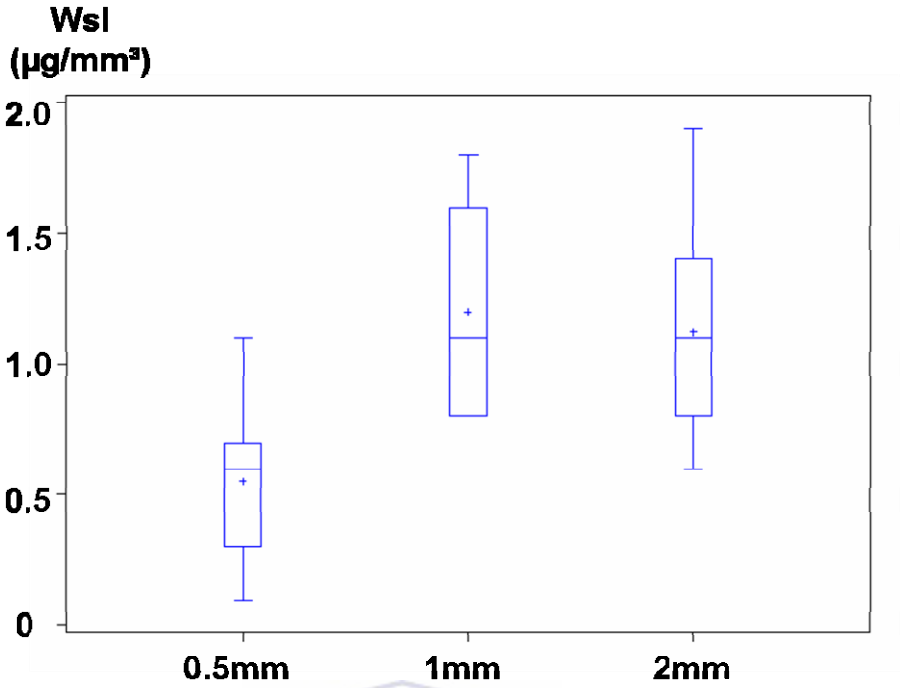


*Figure 11. Box and whiskers plot for the water solubility in µg/mm³ of the 3 thickness groups. Wsl = water solubility.*

Figure 11 demonstrates the opposite to figure 10. The 0.5 mm group has the lowest level of Wsl, while the 2 mm and 1 mm group, again, grouped closely together, had the highest Wsl.

Figure 12 shows the distribution of Wsp over thickness. Thickness influences Wsp and Wsl: solubility increases as thickness increases; sorption decreases as thickness increases.

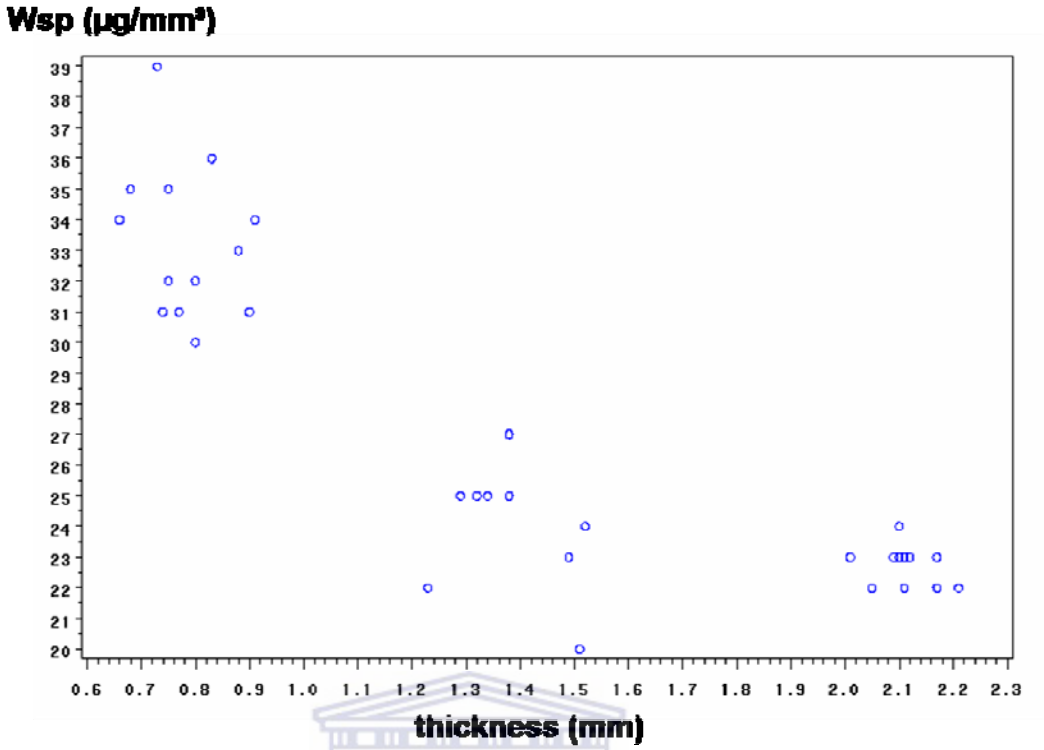


*Figure 12. Scatter plot of water sorption in µg/mm³ over the thickness of the specimens. Wsp = Water sorption.*



### 3.5. Influence of temperature on flexural strength

To test the influence of water immersion temperature on flexural strength, the 4 groups of specimens, used for temperature testing, were used in the test. Table IX shows the summary statistics.

| FLEXURAL STRENGTH | | | | |
|---|---|---|---|---|
| | *22ºC* | *37°C* | *55ºC* | *70°C* |
| Maximum | 108.20 | 107.68 | 116.23 | 122.60 |
| Minimum | 89.13 | 94.48 | 92.05 | 85.90 |
| Median | 100.60 | 98.02 | 102.80 | 98.16 |
| Mean | 99.94 | 99.07 | 102.39 | 99.55 |
| Std Dev | 5.870 | 3.838 | 7.145 | 9.930 |
| N obs | 14 | 12 | 14 | 13 |

*Table IX. Descriptive statistics for the flexural strength in MPa of the 4 temperature groups.*

The Median test for comparing the median strength for each temperature group, shows no significant difference among the groups (p = 0.48). Since no overall significant differences were found, there was no need to look at pairwise comparisons.



The 37ºC group consisted of only 12 specimens due to 1 specimen fracturing when it was being prepared into strips for flexural testing and 1 specimen's data being lost due to equipment failure during testing. The 70ºC group only contained 13 specimens in the results as 1 specimen had fractured during the initial preparations for sorption and solubility testing. The one disk from the 70ºC group that was excluded in sorption and solubility testing due to not reaching a constant mass was included in flexural strength testing.

The data is presented in a box and whiskers plot in figure 13.

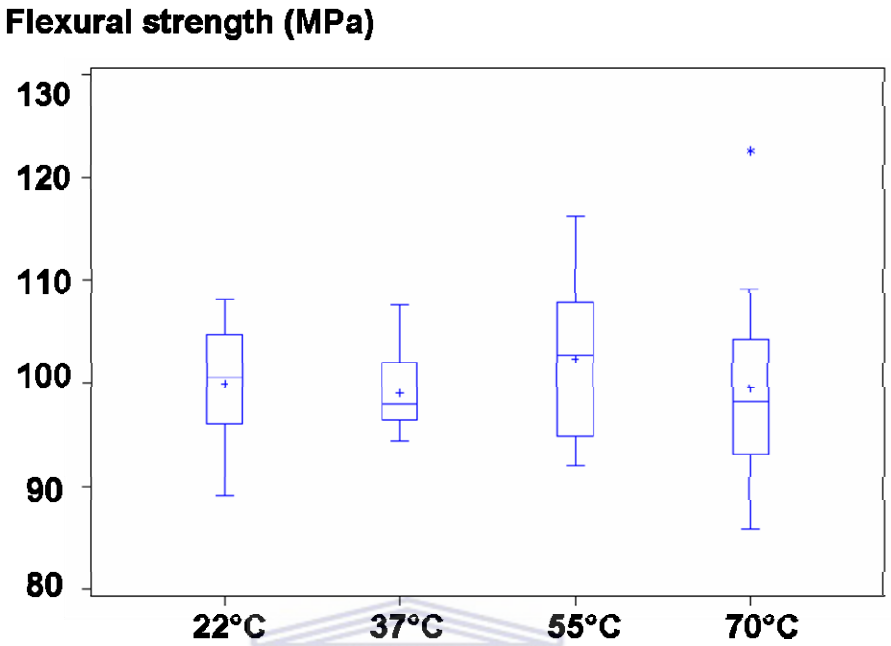


*Figure 13. Box and whiskers plot for the flexural strength in MPa of the 4 temperature groups.*



One outlier can be observed in the 70ºC group. No abnormal width or height was observed in the specimen. All specimens were prepared from the same batch of material and subjected to standardized testing procedure. The outlier did not have abnormal sorption or solubility values. Therefore, the outlier was not excluded from the data set as it might occur in the clinical setting.

### 3.6. The influence of post-polymerization treatment on the cytotoxicity of a denture base resin.

The two post-polymerization treatments, to be tested for cytotoxicity, were polishing and water temperature. A third group consisted of specimens not

subjected to any post-polymerization treatment. The test medium, not subjected to any disk, was used as the control group, acting as a negative control.

Optical density values were obtained at regular intervals. High optical density value readings represent high cell viability.

A preliminary test was performed to confirm the cytotoxicity test procedures and investigate cell viability, over time. Density values were obtained using eluates exposed to the different groups for 24 hrs, and 1, 2, 3 and 4 weeks. Using four disks per group, the average of three disk readings was used as an observation in data analysis to obtain twelve readings per group. This brought the number of observations in the raw data to four values per group. The mean of these 4 mean density values, over time, are plotted in figure 14. Table X shows the summary results for the preliminary test. The analysis was a 2-factor study with one factor being *group* and the other being *time*. The Means procedure was used in data analysis.

| Time | N obs | Variable | Mean | Std Dev | Minimum | Maximum |
|---|---|---|---|---|---|---|
| 24 Hours | 4 | No treatment | 1.076 | 0.076 | 1.162 | 1.321 |
| | | Polished | 1.319 | 0.111 | 1.161 | 1.423 |
| | | Hot water | 1.293 | 0.098 | 1.200 | 1.429 |
| | | Medium | 1.365 | 0.082 | 1.277 | 1.456 |
| Week 1 | 4 | No treatment | 1.220 | 0.156 | 0.986 | 1.318 |
| | | Polished | 1.245 | 0.180 | 0.986 | 1.383 |
| | | Hot water | 1.231 | 0.117 | 1.068 | 1.318 |
| | | Medium | 1.325 | 0.053 | 1.263 | 1.386 |
| Week 2 | 4 | No treatment | 1.488 | 0.055 | 1.419 | 1.542 |
| | | Polished | 1.356 | 0.107 | 1.247 | 1.490 |
| | | Hot water | 1.258 | 0.057 | 1.177 | 1.310 |
| | | Medium | 1.441 | 0.067 | 1.368 | 1.503 |
| Week 3 | 4 | No treatment | 1.086 | 0.109 | 0.941 | 1.195 |
| | | Polished | 1.051 | 0.090 | 0.938 | 1.132 |
| | | Hot water | 1.062 | 0.062 | 0.965 | 1.113 |
| | | Medium | 1.077 | 0.042 | 1.023 | 1.115 |
| Week 4 | 4 | No treatment | 0.692 | 0.046 | 0.652 | 0.758 |
| | | Polished | 0.740 | 0.069 | 0.678 | 0.831 |
| | | Hot water | 0.727 | 0.122 | 0.618 | 0.861 |
| | | Medium | 0.957 | 0.070 | 0.897 | 1.044 |

*Table X. Descriptive statistics of the cell viability measurements for the preliminary cytotoxicity test over a time period of 24 hours up to 4 weeks for the different test groups.*

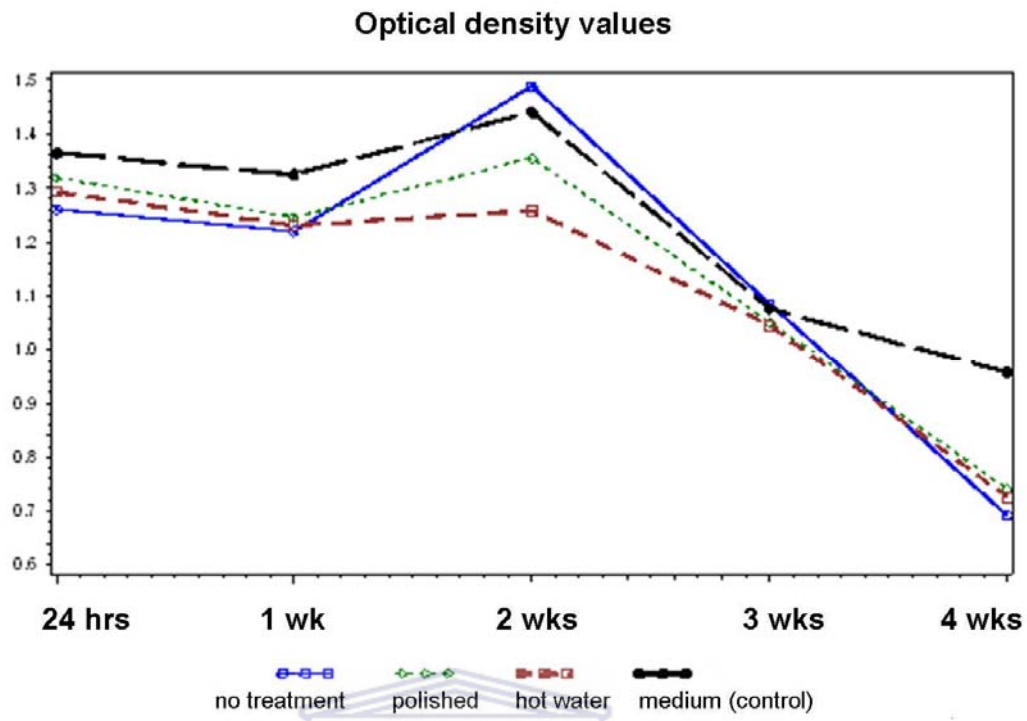


*Figure 14. Graph of the means of the optical density values for the 4 groups over time.*



Optical density values are generally stable from the first 24 hours up to 1 week. For week 2, peak optical density was measured compared to the 24 hours values, representing peak cell viability, for all groups, except for the hot water group. Eluates retrieved after 2 weeks of exposure to the specimens, showed lower optical values for the hot water group than after 24 hours. The optical density values, measured for weeks 3 and 4, were clearly lower for all groups, including the control group, representing strongly diminished cell viability. The medium control reading from week 3 to week 4 was more stable, compared to the test groups. A close grouping of the 3 test groups was present for weeks 1, 3 and 4.

In the second cytotoxicity test, more attention was paid to the first 48 hours. Nine specimens per group were chosen. For this experiment, triplicate density readings per specimen were used to calculate the mean density value for each specimen of each group. The data was analysed using the Means procedure. The descriptive data for the different groups at 24 and 48 hours are shown in Table XI.

| | 24 Hours | | | | 48 Hours | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated | Polished | Hot water | Medium (control) | Untreated | Polished | Hot water | Medium (control |
| Maximum | 0.704 | 0.638 | 0.645 | 0.712 | 0.684 | 0.699 | 0.677 | 0.813 |
| Minimum | 0.576 | 0.558 | 0.574 | 0.559 | 0.554 | 0.637 | 0.591 | 0.636 |
| Median | 0.642 | 0.582 | 0.591 | 0.050 | 0.637 | 0.651 | 0.653 | 0.693 |
| Mean | 0.636 | 0.588 | 0.591 | 0.668 | 0.639 | 0.661 | 0.646 | 0.712 |
| Std Dev | 0.038 | 0.022 | 0.024 | 0.051 | 0.043 | 0.021 | 0.027 | 0.051 |
| N obs | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |



*Table XI. Descriptive statistics for density readings of the 4 different groups for 24 and 48hours.*

The analysis was a two-factor study, with one factor being the group and the other factor being time. The interaction between these factors was found to be significant, indicating that the effect of the groups varied by time (and vice versa). Consequently, group comparisons (table XII, table XIII, and figure 15) and time comparisons (table XIV and figure 16) were performed at fixed intervals (24 and 48 hours) for each group.

The tables XII, XIII and XIV show (underlined) p-values <0.01 which can be considered as being significantly different.

| Group comparison | Lower value | Upper value | Standard error | P |
|---|---|---|---|---|
| Hot water – medium | -0.104 | -0.034 | 0.172 | 0.0003 |
| Hot water - polished | -0.024 | 0.0467 | 0.172 | 0.5076 |
| Hot water - untreated | -0.072 | -0.002 | 0.172 | 0.0390 |
| Medium - polished | 0.046 | 0.116 | 0.172 | <.0001 |
| Medium – untreated | -0.003 | 0.067 | 0.172 | 0.0718 |
| Polished - untreated | -0.084 | -0.014 | 0.172 | 0.0081 |

*Table XII. Group comparisons of density readings for 24 hours. "Medium" represents the control group. P = probability.*



| Group comparison | Lower value | Upper value | Standard error | P |
|---|---|---|---|---|
| Hot water – medium | -0.101 | -0.031 | 0.172 | 0.0006 |
| Hot water - polished | -0.0495 | 0.021 | 0.172 | 0.4120 |
| Hot water - untreated | -0.028 | 0.042 | 0.172 | 0.6782 |
| Medium - polished | 0.016 | 0.087 | 0.172 | 0.0053 |
| Medium – untreated | 0.038 | 0.109 | 0.172 | 0.0002 |
| Polished - untreated | -0.014 | 0.057 | 0.172 | 0.2204 |

*Table XIII. Group comparisons of density readings for 48 hours. "Medium" represents the control group. P = probability.*

| Group | Lower value | Upper value | Standard error | P |
|---|---|---|---|---|
| Hot water | -0.082 | -0.013 | 0.017 | 0.0089 |
| Medium | -0.079 | -0.009 | 0.017 | 0.0143 |
| Polished | -0.108 | -0.039 | 0.017 | 0.0001 |
| Untreated | -0.038 | 0.032 | 0.017 | 0.8609 |

*Table XIV. Time comparisons of density readings for 24 and 48 hours for each group. "Medium" represents the control group. P = probability.*

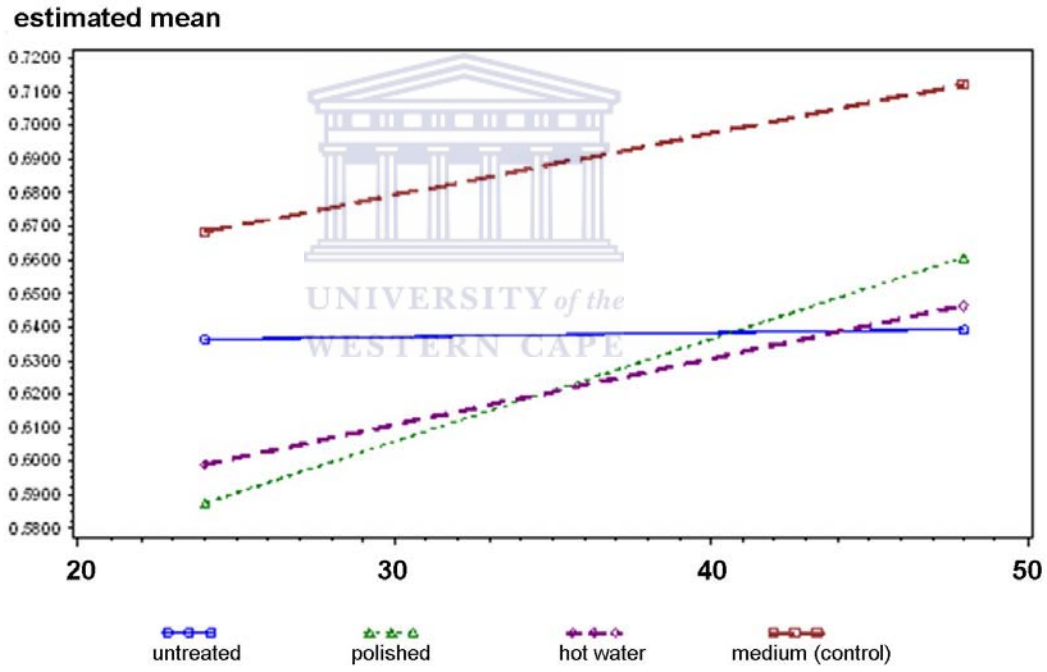


*Figure 15. Graph of the estimated mean density readings over time (hours) for all the groups.*

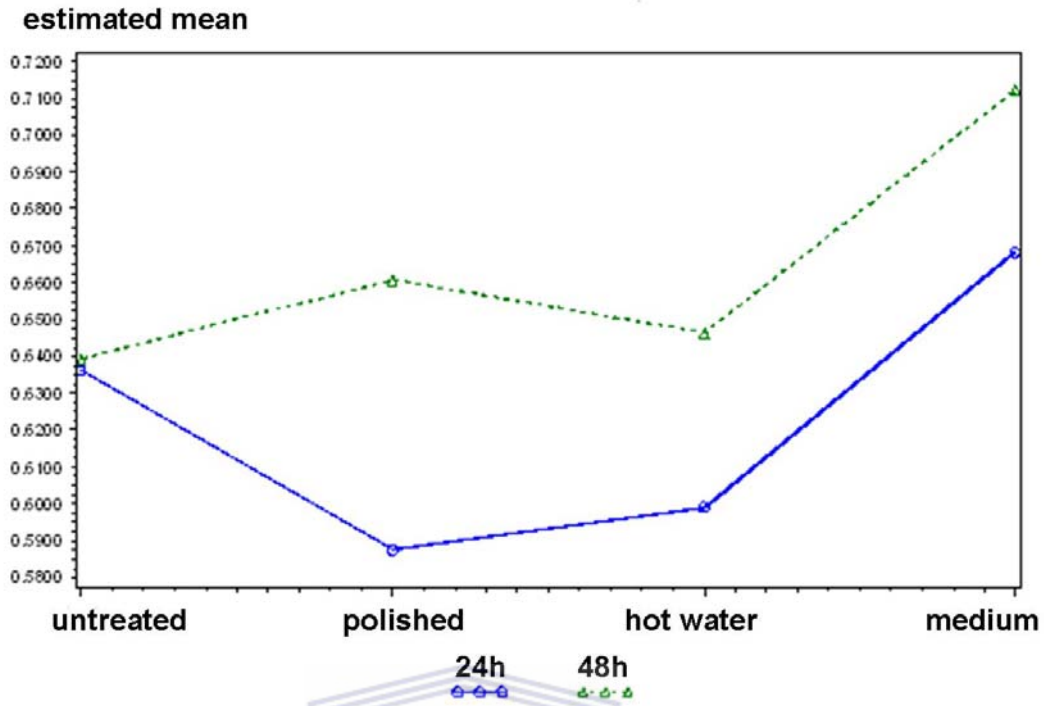


*Figure 16. Graph of the mean density readings for all the groups at 24 and 48 hours.*

The two timelines on the above graph are inserted for ease of comparison, it is of no other analytical value. The values for 24 and 48 hours are plotted per group, for comparison.

# CHAPTER 4

# DISCUSSION

## 4.1. Introduction

This study investigated the influence of polishing, mixing ratio, thickness and water-soaking temperature on the sorption and solubility of a type 1, class 1 denture-base polymer. The flexural strength of specimens was also tested after water immersion at different temperatures. Those post-polymerization treatments that significantly decreased solubility, were applied to specimens to be used for *in vitro* cell viability testing, by means of the MTT- assay.



## 4.2. Solubility and sorption

### 4.2.1. Introduction

Testing for sorption and solubility was based on the ISO 1567 standard. This standard requires that specimens be weighed 24-hourly until a conditioned mass is reached, after manufacturing, and, after seven days of water immersion. A conditioned mass is reached for a specimen when the difference in weight is equal to or less than 0.2 mg, between two consecutive readings. When a specimen reached a stable mass for the first time between two readings, this was considered to be its conditioned weight.

The time required to complete each experiment differed (table 1), because the thickness of the specimens for each experiment differed. The thickness of the specimens for the polishing experiment was 2 mm, for the mixing-ratio experiment 1 mm and for the temperature experiment, 0.5 mm. Not all specimens within each group reached their conditioned mass on the same day. For example, specimens for the polishing experiment reached M1 over a period of seven days, and M3 over a period of four days. Sadamori *et al*. (1997) also reported that the period necessary to obtain constant weight depended on the thickness of the specimens.

Even though the number of specimens prepared for each group, within each experiment, was equal, the number of observations per group, as reflected in the results, differs. Some observations were removed from the results, because of the specimens having failed to reach the "conditioned mass" as specified by the ISO 1567 standard for sorption and solubility testing. Specimens had to reach a conditioned mass twice: once before water immersion (M1) and a second time after being soaked in water (M3).



The group sizes for each experiment differed. They varied between twelve and fourteen disks per group. Initially, when the first experiment (influence of polishing on sorption and solubility) was done, a group size of twelve was chosen as a unique number of observations. When complications occurred, such as insufficient drying of some of the specimens that needed to be removed from the

experiment, the group sizes for subsequent experiments were increased to compensate for possible complications.

The ISO standard recommends the manufacturing of disks with a thickness of 0.5 mm. The thickness of the specimens for testing polishing and mixing ratios was 2 mm and 1 mm respectively, for reasons that will be explained later. Therefore, conclusions, in terms of ISO standard compliance, will not be made for these two tests.

Even though material and equipment was handled under strictly controlled conditions, the presence of outliers was a general, though infrequent, occurrence. This raised concerns to what would happen in a clinical environment where materials and equipment are not necessarily handled under the same, strictly controlled conditions.



Possible negative consequences of water sorption, are dimensional changes and loss of mechanical strength. Within limits, expansion, linked to the absorption of water compensates for polymerization shrinkage and may lead to a better fit of the prosthesis (Monfrin *et al.,* 2005). However, others claim that these changes are considered too small to be of clinical significance (Dixon *et al.,* 1992). Of more concern, is the loss of strength of polymer resins due to water sorption. Water sorption leads to the relaxation of internal polymerization stresses and contributes to fatigue (Ristic & Carr, 1987; Dhir *et al.*, 2007). Cracks may develop and,

ultimately, denture fracture may occur. Therefore, it would be advantageous to limit water sorption as much as possible.

As stated by Doğan *et al*. (2005), water sorption can be decreased by increasing polymerization time and temperature. This also results in less residual monomer. Less residual monomer may decrease the cytotoxicity of the material. The effect on cytotoxicity will be discussed in more detail in 4. 4.

### 4.2.2. The influence of polishing on sorption and solubility

It is important to polish dentures for patient comfort, aesthetics, denture hygiene, prosthesis longevity and to reduce the adhesion of micro-organisms and plaque formation (Ulusoy *et al.,* 1986). By polishing, the denture base is smoothened and surface porosities are removed. Kuhar & Funduk (2005) showed that conventional laboratory polishing of denture bases created the smoothest surface, and, thus, being preferred to chair-side polishing kits and tungsten-carbide bur finishing. The polishing procedure followed for this study, was the technique described in the ISO 1567:1999(E) standard – Denture base polymers, which is a laboratory polishing technique, using pumice and a wet muslin wheel, followed by the use of a polishing compound with an unstitched muslin wheel.



The ISO standard for sorption and solubility testing recommends a thickness of 0.5 mm for the disks. For the purpose of assessing the influence of polishing on sorption and solubility, disks of 2 mm were prepared, instead. The reason for the extra thickness was to reduce the risk of fracture or perforation of the specimens

during polishing. This is probably the reason why the specimens took so long to reach the conditioned state (M1: 8 - 15 days, and M2: 28 - 32 days). This may also have been the reason why three specimens failed to reach a constant mass during conditioning.

For sorption, there was no significant difference between the polished group and the unpolished group ($p = 0.53$). For solubility, the polished and unpolished groups were significantly different, with the polished group being less soluble ($p = 0.0056$). Therefore, the null-hypothesis for this experiment is partially rejected.

From the results, it is indicated that polishing a denture base will have no significant difference on the sorption when it is immersed in water for seven days. But, by polishing a denture base resin, its water solubility is significantly lowered compared to an unpolished denture base.



The results of this study support the findings of the study by Vallittu (1996). He found that the leaching of MMA from auto-polymerizing PMMA was reduced by conventional polishing or by coating with a light-curing resin. Due to the potential toxicity of some of the components leaking from denture base resins, in particular MMA and formaldehyde, one should aim at reducing solubility.

It can be speculated that the difference in the findings between sorption (no significant difference) and solubility (significant difference) between the two

groups is probably due to different chemical processes, the polarity and size of different molecules involved in the processes of sorption and solubility.

Because polishing did not reduce sorption of the denture base material, the degradation of the material due to sorption, is not expected to be reduced by polishing. Since polishing reduces solubility, the polishing of dentures should be done *after,* and not prior, to water soaking, in order to obtain the maximum pre-leaching of possibly harmful substances from the dentures before delivery.

### 4.2.3. The influence of different mixing ratios on sorption and solubility

Three different mixing ratios were compared to investigate the influence of mixing ratio on sorption and solubility. Besides the recommended mixing ratio, two additional ratios of 25% more and 20% less powder than the prescribed ratios were used for this experiment. A pilot study determined that these ratios were the practical limits in terms of handling properties: ratios outside these percentages made the dough difficult to work with. Again, the thickness of the disks, for this experiment, was modified. Instead of the ISO-described 0.5 mm, the thickness of the disks was 1 mm. This was done to compare the influence of different thicknesses on sorption and solubility which will be discussed in 4.2.5.



For sorption, there was no difference among the three groups ($p = 0.34$). For solubility, small, but insignificant, differences were measured among the three groups ($p = 0.0683$).

Therefore, the null-hypothesis is accepted for the mixing ratio experiment.

Solubility is often related to the leaching of residual monomer. It may be speculated that a higher liquid content in the mixing-ratio results in a higher residual-MMA content. This MMA could then leach out over time. However, this study did not find a higher solubility for different mixing ratios. These results are in line with the results of Kedjarune *et al*. (1999), who stated that resin with the lowest residual monomer content also released the smallest amount of MMA, but also, that resins with higher monomer content, may not necessarily release more MMA.

This study did not analyse the residual MMA content in the specimens made from different mixing ratios. It is, therefore, not known if different ratios resulted in different residual monomer contents. This should be considered a study limitation and could be researched further. However, within the limitations of the test, it can be concluded that, for this material, mixing ratios did not have an influence on the solubility of the polymerized product.



It is not recommended to extrapolate results of this study to other materials. Kedjarune *et al*. (1999), stated that the amount of residual monomer not only depends on the amount of monomer in the mixing ratio, but also varies according to brands, type of polymerization and processing methods. In my study, the type of material and polymerization procedure was identical for all the groups, with the only variable being the mixing ratios. Further research could be done, using the

same material, but changing polymerization time, to determine if a reduction or an extension of the polymerization cycle influences residual MMA and solubility.

The specimen responsible for the outlier in the liquid group for solubility (figure 7), was identified. This specific specimen's second reconditioning mass (M3), after water immersion, was less than the first conditioned mass (M1), before water immersion, compared to the other specimens in the liquid group. This occurred in only one specimen out of a group of twelve. This could be the result of an unidentified manufacturing error, or this could be a recurring feature. A more accurate indication of the prevalence of this phenomenon could be assessed, should the test groups be larger. If this excessive weight loss during the second conditioning process could happen in a controlled test environment, it could also happen in a clinical environment. Therefore, future research could establish if this is a recurring feature for specimens prepared with a higher liquid content. It can be speculated that, because of the higher liquid content of the mixture, more residual monomer was present and that during water immersion, this monomer was released. During the second conditioning process the disc mass was less because of the loss of the residual monomer.



In a busy laboratory, it probably happens more often that more monomer (liquid) is incorporated into the mixture in an effort to increase working time and to process more dentures simultaneously, than the other way round (adding more powder). However, within the limitations of the mixes under investigation, none of the two scenarios outside the control had an effect on the sorption and

solubility of the polymerized product. As speculated by Jerolimov *et al.* (1985), the choice of polymerization cycle has a greater influence on the MMA level in PMMA, than the powder/liquid mixing ratio used. It can be concluded that, within limits, it is acceptable to alter the mixing ratios. Increased polymerization time can be used to compensate for altered mixing ratios, within limits, as demonstrated by Kedjarune *et al*. (1999).

Most studies, found in literature, on the effect of mixing ratios on sorption and solubility, have been performed using auto-polymerizing resin. No literature could be found dealing with studies on the sorption and solubility of heat-polymerized denture base resin.



### 4.2.4. The influence of water immersion temperature on sorption and solubility

Tsuchiya *et al.* (1994), reported that pre-leaching acrylic denture base materials in water reduces the subsequent leaching of formaldehyde and MMA. Vallittu *et al.* (1995) and Bayraktar *et al.* (2004), also reported that storing dentures in distilled water is a simple, but effective, method of reducing its residual MMA content. All these studies used a water temperature of 37°C.

Since diffusion is influenced by temperature (Vallittu *et al.,* 1995), the influence of water temperature on sorption and solubility, was investigated, by submerging four groups of 14 specimens each into water at different temperatures (22, 37, 55 and 70 ºC). For sorption, doing pairwise comparisons, all pairs were significantly

different, except the 22ºC - 37ºC pair. For solubility, all pairs were significantly different, except the 37ºC - 55ºC pair and the 55ºC - 70ºC pair.
Therefore, the null-hypothesis is partially rejected.

Because the immersion of denture-base material in hot water reduces water sorption, it is recommended that dentures be soaked in water at temperatures higher than 37°C, and, preferably, at 70°C, because this temperature is associated with the highest reduction in sorption. This temperature also increases solubility and the release of monomer before delivery of the denture to the patient. Since polishing reduces the solubility of a denture base acrylic, pre-soaking should be done after polymerization, but before polishing.



The box and whisker plots of sorption and solubility results for this experiment (figures 8 and 9), resemble an inverse proportional relationship of sorption and solubility to each other: higher soaking temperatures lead to a lower sorption and higher solubility.

It is difficult to explain this phenomenon. It may be speculated that the higher temperature leads to continued polymerization, creating a denser network of polymer chains with less access for water molecules. Using high-performance liquid chromatography, Vallittu *et al.* (1995), analyzed the monomer content of chemically- and heat-polymerized specimens stored at 22°C and 37°C and found that less monomer was present in specimens stored at the higher temperature. On the other hand, the lower monomer content in specimens stored at higher

temperatures makes it difficult to explain the higher solubility at higher temperature. It may be speculated that the higher temperatures induce a larger proportion of the relatively lower monomer content to leach out, compared to less leaching of more monomer present in the specimens stored at lower temperatures. No literature was found that adequately explains this inverse relationship of sorption and solubility in terms of temperature. This could be a field for further investigation.

Whatever the explanation, it remains advantageous to immerse the polymerized denture base in hot water, because it decreases sorption and the possible associated degradation. It also increases solubility and the possible release of monomer prior to placement of the denture.



Some outlier values can be observed in the box plots (figures 8 and 9). As mentioned previously, figure 8 may appear to have two outliers. Observing the data of the 70°C group, the outliers as plotted are the maximum and minimum value specimens. Due to the close grouping of the 70°C test group, they are not actually outliers when observed in the context of the entire test.

In figure 9, two outliers can be observed for solubility. These outliers represent two specimens (one in each of the 55ºC and 70ºC group) that had lost more in weight after the second drying-out process (M3), after water immersion, than they had weighed, initially, during the first conditioning (M1), before water immersion. All specimens were treated identically during processing and conditioning. No

reason for this behaviour difference could be found, related to errors in the testing procedure. Therefore, the specimens were kept in the dataset.

The occurrence of M1>M3 for solubility was also observed in the mixing ratio experiment in one disk from the liquid group (group with a higher monomer content).

The solubility values of the two different experimental groups (water immersion and mixing ratios) cannot be compared, since they are not even in thickness. However, looking at the solubility of the different experimental groups, the outliers were observed in each of the test groups.

With the speculation being made, in the liquid group, that the phenomenon was due to the residual monomer released into water, the 55ºC and 70ºC specimens can be included in this speculation, because of the higher monomer release observed in this group. Another factor to be taken into consideration when comparing the two experimental groups, is that the thinner disks in the temperature group will have a higher monomer level than the thicker disk of mixing ratios. This finding, according to Sadamori *et al.* (1994), states that a thin heat-polymerized resin disk will have higher levels of residual MMA than thicker disks.



Studies have shown that the longer the period of immersion in water, the lower the content of monomer in the specimens. The Vallittu (1995) study used eight weeks of water immersion at two different temperatures (22°C and 37°C). However, eight weeks is not a practical time lapse in modern dentistry. My study

only investigated a single-time period (seven days, as recommended by ISO for sorption and solubility testing), but included higher temperatures. The time/temperature relationship in sorption and solubility testing, or monomer content analysis, could be explored in subsequent studies. Paragraph 4.4 will discuss the influence of time on the *in vitro* cytotoxicity of a denture base resin.

#### 4.2.4.1. Comparing the results with ISO compliance

In the above experimental group, the thicknesses of the disks were prepared according to the ISO specification for sorption and solubility testing, which is 0.5 mm thick. According to the ISO 1567, the value for sorption should not exceed 32 µg/mm³ and, for solubility, the accepted value should not exceed 1, 6 µg/mm³ at 37°C. ISO also specifies that, for sorption, at least four out of every five specimens should comply with the requirements. In my study, the individual values of seven of the fourteen sorption specimens comply with ISO specifications. Because ISO uses five specimens for this specification and this study had fourteen specimens, the values were converted into percentages to be comparable to each other. This resulted in the material being right in the middle, between absolute failure and the re-running of the test to see if it does comply during the second trial. For solubility, all the specimens complied with the ISO requirements.



The manufacturers' data sheet for the product claims solubility values of 0.11 µg/mm³ and sorption values of 22.5 µg/mm³. When comparing these values of the material with the ISO values, the values of the manufacturer are substantially

lower. When the values of the tested material are compared to the values achieved in this study, it is interesting that only one specimen complied with the manufacturers' data for solubility, and none of the specimens complied in the case of sorption.

### 4.2.5. The influence of thickness on sorption and solubility

Denture thickness is variable, differing for each patient, depending on individual anatomy. Three different thicknesses were investigated, i.e., 0.5 mm, 1 mm and 2 mm, for their sorption and solubility properties. For both sorption ($p<0.0001$) and solubility ($p = 0.0051$), significant differences between the 0.5 mm-groups and both the 1 mm- and 2 mm-groups, were found, but not between the 1 mm- and 2 mm- groups. Observing the box plots (figures 10 and 11), an inverted relationship between sorption and solubility as it relates to specimen thickness is evident.



The null-hypothesis is rejected, except for the 1 mm – 2 mm pair.

This study did not have a group of specimens with a 1.5 mm disc thickness. The reason for this was that the investigation of the influence of thickness on sorption and solubility was not part of the original methodology. However, since data were available on the different specimen thicknesses, it was decided to statistically analyze the available data, retrospectively. The inclusion of a 1.5 mm-thickness group could have given a more complete picture on the sorption and solubility behaviour.

Sadamori *et al*. (1994), reported that thinner specimens of a heat-polymerized resin have a higher level of MMA content than thicker specimens. According to Austin & Basker (1980), this is explained as follows: "*the heat evolved during heat-polymerized acrylics during polymerization causes the thicker specimens to reach higher temperatures, resulting in a greater degree of polymerization and a corresponding reduction in the amount of residual monomer*". As speculated earlier, the leaching of residual monomer might create voids in the polymer that are taken up by water during water immersion. Therefore, high residual monomer is associated with higher sorption values, as seen in this experiment, where thinner specimens, indeed, have a higher mean sorption value than the thicker specimens.

The theory of Austin & Basker (1980), does not explain the results of this study, namely, that the solubility of thicker specimens is higher than that of the thinner specimens. Since their monomer content is lower, as shown by Sadamori *et al.* (1994), and, since monomer is regarded as the main leaching substance, one would expect a lower solubility value as well.

Since solubility is higher for thicker specimens, the prevention of unnecessarily thick denture bases is a recommendation.

Using gas-liquid chromatography, Sadamori *et al*. (1994), reported that thinner specimens have higher residual monomer content than thicker specimens. In 1997, Sadamori *et al.* reported that the weight increase for thicker specimens (3 and 5 mm) was greater than for thinner ones (1 mm), after water immersion. This is in contrast with the results of this study, regarding water sorption. In general, the

thicker specimens (1 mm and 2 mm) had lower water sorption (figure 10). However, it must be taken into consideration that Sadamori *et al*. (1997), didn't follow the ISO standard procedures and used a different heat-polymerized denture base resin brand.

A universal optimal denture base thickness is difficult to recommend, because of clinical requirements in terms of denture support, tissue support and individual anatomic variations. From the perspective of biocompatibility, which is arguably more important than the mechanical properties of prosthesis, thinner, rather than thicker, denture bases are recommended.



**4.2.6. Solubility and sorption: concluding remarks**

The results of my study have the following clinical implications:

1. The immersion of dentures, after manufacturing, in water at 55 ºC (or 70 ºC for these two groups are not significantly different from each other), before delivery of the dentures, reduces sorption and increases solubility to allow for the pre-leaching of potentially harmful substances.
2. Since polishing reduces solubility, polishing should be performed after pre-leaching.
3. Avoid unnecessarily thick denture bases during denture fabrication.

### 4.3. Flexural strength

Dentures function in a wet environment and are often stored in water when not in use. The influence of water-soaking temperature on the flexural strength was investigated. Disks from the four temperature groups in 4.2.4. were modified into strips for flexural strength testing. The groups had been immersed in water at 22, 37, 55 and 77°C for seven days during sorption and solubility testing. Before flexural strength testing, the disks were again immersed in 37ºC distilled water for another 50 hours, as described by the ISO on flexural strength testing. One disk of the 37ºC group was lost when it fractured during the conversion form a disk into strips, and another specimen's data was lost due to equipment failure during testing. The 70ºC group also only contained 13 specimens in the results, as one specimen fractured during the preparations for sorption and solubility testing.



There were no significant differences in flexural strength among the four temperature groups. Therefore, the null-hypothesis stating that there is no difference in *flexural strength* among different water immersion temperatures of a denture base polymer is accepted.

Lassila & Vallittu (2001), reported that the storage of a hypo-allergenic denture base resin polymer, in water, reduces its flexural strength, compared to no water storage whatsoever. My study only compared the soaking temperatures with each other and did not include a group of specimens that were kept dry. Water immersion could alter the influence on flexural strength testing when compared to water immersion and no water immersion tests, due to sorption and solubility

changes, as well as possible residual monomer release. Within the limitations of my study, it may be concluded that the water temperature had no detrimental effects on the flexural strength of the tested denture base material. Since immersion in hot water reduces solubility, this practice could, therefore, be supported and recommended.

The specimens observed as outliers for water solubility in the temperature experiment were identified, and they did not have an exceptional flexural strength reading. This exceptional solubility did not have an influence on their flexural strength. However, since this was observed for only two specimens (one in each of the 55ºC and 70 ºC group), future investigation should be done with large groups.



Water sorption by acrylic resins causes dimensional instability and fatigue, which can lead to crack formation and, subsequently, to fracturing of a denture. The flexural strength of denture bases is an important mechanical property. The longevity of dentures depends, in part, on the flexural strength of the acrylic resin (Beyli & Von Fraunhofer, 1981). This study only examined the influence of different water-storage temperatures on flexural strength. For future research in this area, a combined test, investigating the effect of different water immersion temperatures, as used in this study and no water immersion, could be conducted.

## 4.4. Cytotoxicity

### 4.4.1. Introduction

Dental materials must be investigated to ensure their biocompatibility with intra-oral tissue. Testing dental materials by using cell-culture methods, avoids human and animal testing, which may pose ethical problems. *In vitro* cell tests are relatively simple to perform, reproducible, and often cost effective. Being *in vitro*, such tests are more easily controlled than *in vivo* tests.

### 4.4.2. Cytotoxicity testing in general

For dental materials, the $^{3}$H thymidine and MTT test are frequently used for *in vitro* cytotoxicity testing. Although the $^{3}$H thymidine test is recognized as the most sensitive for resin cytotoxicity testing (Jorge *et al*., 2004) the MTT test was used for this study, because staff with experience in this particular test were available at the Research Institute of the Faculty of Dentistry at UWC. Also, the $^{3}$H thymidine test requires special, expensive equipment and generates radioactive waste. The scope of this study did not justify these disadvantages. The limitation of the MTT assay is that it measures cell viability by means of colour density measurements of the preparations. True levels of cytotoxicity can only be determined by cell apoptosis and necrosis studies (Cimpan *et al.,* 2000).



### 4.4.3. MTT test

The post-polymerization treatment factors that influenced sorption and solubility most significantly in this study, were polishing and water temperature. Different thicknesses of disks also showed to be a factor in sorption and solubility, but this

factor could not be tested together with the other two groups within the same MTT assay, because of a different disk mass and extraction-medium volume ratio during eluate preparation, compared to the other two groups.

In my study, eluates extracted from disks were used to test the viability of a cell culture. Cimpan *et al*. (2000) reported that the deleterious effects on cells were stronger, if cells were plated directly onto the polymer disk, compared to using eluate extracts. Therefore, it is speculated that in the *in vivo* process, where dentures are in contact with the oral mucosa, the effect of direct contact may be more severe than the results of tests using eluates.



The test specimens were not subjected to ultrasonic cleaning before testing, as done in other studies (Campanha *et al*., 2006; Jorge *et al.,* 2004). There was a risk that the fluid and heat generation in the ultrasonic bath may lead to pre-leaching. Instead, the specimens were subjected to ultraviolet exposure for 20 minutes prior to testing. Besides the advantage of a dry environment, Sheridan *et al.* (1997) reported that ultraviolet light did not have an influence on the polymerization of the specimen.

### 4.4.4. The results of post-polymerization treatments on cytotoxicity

The material used in this study was a rapid-heat-polymerizing resin. According to Vallittu *et al*. (1995), rapid-polymerizing resins have an additional activator leading to a higher degree of monomer conversion, compared to conventional resins with a long polymerization cycle.

In the preliminary investigation, the cytotoxic effect of the denture base material was observed over a period of four weeks (table X). One-day and 1-week-long exposures to eluates in all the groups had no significant effect on the optical density values, suggesting no major effects on cell viability for these two time periods. This is contrary to expectations, since the sorption and solubility study, of this paper, shows that polishing significantly reduces solubility and that pre-leaching in hot water increases solubility which would suggest lower residual monomer within the disks and higher cell viability, compared to the no-treatment groups.

Two-week long exposure to eluates showed an increase in optical density values for all groups, except for the no-treatment group. This result is also contrary to expectations. The disks in this group were not pre-leached or polished before testing. Therefore, it would have been expected that this group would have had a higher monomer content, higher solubility and therefore a higher cytotoxicity level. Lefebvre *et al*. (1994*)*, using the MTT assay and $^{3}H$ thymidine-incorporation test on oral epithelial cells, observed similar stimulation of cell activity for some materials at certain time intervals. They speculate that this stimulation may be the result of a compensatory response of cell-enzyme activity to resin-associated cytotoxicity.

At week three, a steady decline in cell viability was observed for all four test groups (figure 15). This included the group that that had only been exposed to the

medium. This suggests that at three weeks of cell culturing, there was a general decline in the viability of the cell culture that is not attributable to a possible cytotoxic effect of the material.

The four-week exposure to eluates again showed lower cell viability for all four groups. With all three of the test groups having the lowest cell viability, it suggests that the eluates of the three test groups that had been exposed for a longer time, contributed to additional loss in cell viability. All three test groups, regardless of any post-polymerization treatment, were more cytotoxic than the control medium that had not been exposed to any disks at four weeks.

The fluctuations of the test groups, compared to the control medium at different time intervals, are difficult to explain, and require further investigation.



A limitation of this initial cytotoxicity test was the group size.

For the second test, the groups contained nine disks each. Again, the mean of triplicate eluate readings for each disk was used, resulting in nine readings for each group. Since it is reported that residual monomer leaching is higher in the first days following the manufacturing of the denture (Tsuchiya *et al.,* 1994), this second test concentrated on measuring the cell viability after eluate exposure of 24 and 48 hours.

This time, the analysis was focused on the influence of the post-polymerization in terms of the two time intervals, and the influence of time on the cell viability levels of the four groups. Group comparisons at the two time intervals (figure 15) and time comparisons for each group (figure 16), were performed. The interactions between these factors were found to be significant.

### 4.4.5. Group comparisons for each time interval

Twenty-four-hour exposure showed the following cell viability responses (Table XII, figure 15): the control medium had the highest cell viability readings. This was to be expected, the control medium not having been exposed to the denture base material. It had significantly higher cell viability than the hot-water and polished group, but not higher than the untreated group. This is somewhat unexpected, since the solubility study showed that pre-leaching in hot water increased solubility resulting in less residual monomer in the disks, and that polishing disks reduced their solubility. However, the 24-hour cytotoxicity results showed that the untreated (non-polished, not pre-soaked) group had a significantly better cell viability response, slightly, but not significantly lower, than the control medium. As mentioned earlier, this might be explained by a compensatory response of cell enzyme activity to resin-associated cytotoxicity (Lefebvre *et al*., 1994).

The fact that the 24-hour eluates of the hot-water and polished groups had a lower viability value than the control medium was to be expected: although residual monomer content and leaching is expected to be reduced by these post-

polymerization treatments, it was not enough to limit cytotoxicity lower than the control medium's cell viability.

Forty-eight hour exposure showed the following cell viability responses (table XIII, figure 16): the medium control group again showed the best viability response and this was significantly better than the three test groups. This is to be expected since the medium control was not exposed to the resin. At 48 hours of exposure, the polished and pre-leached groups did not have a lower cytotoxic effect compared to the untreated group. This was not expected, for the same reasons as explained for the 24 hour eluates. However, this time there was no significant difference between the three test groups. It may be speculated that the compensatory response of cell-enzyme activity to resin-associated cytotoxicity is now lower.



**4.4.6. Time comparisons for each group**

The viability of the medium control improved from 24 to 48 hours, but not significantly. The viability readings of the untreated group did not differ between the 24 and 48 hour exposure to eluates. However, taking into consideration that the control medium improved in terms of cell viability, the cell viability of the untreated group may be considered to have slightly deteriorated.

However, the viability readings for the polished and hot water groups improved from 24 to 48 hours. This improvement was significant. The viability readings from 24 hours to 48 hour of these two groups did not differ. This study did not

include a test group that had both post-polymerization treatments (polished and hot-water groups) in one group. It can be speculated that both treatments may have a cumulative effect and lead to an enhanced improvement in cell viability readings. This could be investigated further.

These results obtained from the group that was pre-soaked in hot water are in line with the results from Sheridan *et al.* (1997). They reported that cytotoxicity appeared to diminish as disk immersion time was increased. The greatest cytotoxic effect on cell viability was observed in their study with eluates recovered after 24 hours of disk immersion, and the least cytotoxic effect with eluates recovered after 96 hours of immersion.



It is difficult to compare the results of this study with results that have already been published. The reason for this is the wide variety of material brands, polymerization methods, different cell lines and tests being performed. Cimpan *et al*. (2000) warned that significant differences existed even among brands belonging to the same type of denture base material.

The unexpected fluctuations in cell viability seen in this study and also present in other studies, may be explained by the stimulation and inhibition of cell reactions in response to cytotoxic substances leaching out of the material at different rates (Lefebvre *et al.,* 1994).

Another explanation was presented by Sheridan *et al.* (1997). Using MTS human gingival fibroblasts, they found that 96-hour eluates had less impact on cell

viability than younger eluates. They hypothesised that toxic substances released in the medium were either degradable over time, or formed complexes with other chemicals in the medium altering their cytotoxic potential.

Zissis *et al.* (2008) reported that, using gas-liquid chromatography, a heat-polymerized denture base acrylic polymer showed no significant loss of residual monomer during 38 months' storage in water. However, referring to the Lung & Darvel (2005) concept of equilibrium between polymerization and depolymerization in an open system, monomer might indeed have been released, but the lost monomer was replaced by depolymerisation. In fact, these authors were very outspoken and reported that the issue of this equilibrium is ill-understood and not addressed in the explanation of research results dealing with residual MMA and cytotoxicity. This open system also existed in this study, where some disks were pre-soaked in hot water and all disks were placed in the medium to generate eluates. Due to the continuous replacement of the lost monomer by depolymerisation, this may only temporarily lead to lower monomer content, and may be responsible for some unexpected results.



Most studies concentrate on the harmful effects of residual MMA. However MMA is not the only substance leaking from denture resins. Formaldehyde, although it is present at lower concentrations in eluates (Tsuchiya *et al*., 1994), has been shown to have a higher cytotoxicity than MMA. In addition, it may react with amines and amino acids in the oral cavity to produce bioactive carbolines and related compounds (Yu *et al*., 1988 in Tsuchiya *et al*., 1994). These

compounds may demonstrate different pharmacological behaviour and even have mutagenic potential (Wakabayashi *et al.*, 1983 in Tsuchiya *et al.,* 1994).

Again, referring to Lung & Darvell (2005), in a closed system, such as *in vitro*-testing, MMA production through depolymerisation may not happen at the same rate as *in vivo*, because of the equilibrium achieved in the system.

Kedjarune *et al.* (1999) states: *"In the oral cavity, it is possible that the concentration of MMA released against the oral mucosa under the denture is higher than the salivary content"*. Monomer under a denture is not rinsed away by saliva and may lead to a pronounced topical reaction. Minor irregularities in the fit of a new denture may provide a source of irritation that makes the mucosa more susceptible to MMA or other chemicals (Kedjarune *et al.,* 1999). This could also be expected in the presence of pre-existing denture stomatitis.



**4.5. Limitations**

1. This study investigated only one material, a Type 1, class 1 denture-base resin.
2. Sorption and solubility testing identify weight gain and weight loss. It does not identify the nature of the substance lost or absorbed.
3. The MTT test was performed because of expertise present within the faculty, a limited budget, and the scope of the study not justifying the cost, nor the radio-active waste produced by other tests.

4. Only the influences of the eluate from the specimens were tested on cells. The level of cytotoxicity may be higher when cells are cultured in direct contact with the disk.

5. Influences, like extreme temperatures caused by hot and cold food and fluids, are not taken into consideration. Thermo-cycling can be used to determine this. The effect of masticatory forces on the denture is also not taken into consideration.

6. Pre-existing pathology, such as denture stomatitis or trauma from dentures may exacerbate the cytotoxic effect of harmful substances released from denture bases.

### 4.6. Clinical relevance

From the *in vitro* tests in this study the following recommendations are clinically relevant:



- ✓ In the technical laboratory, within the tested limits, mixing ratios may be altered without affecting sorption or solubility properties of the material.
- ✓ Denture base thickness varies, depending on individual oral anatomy. It is difficult to recommend an ideal denture thickness: for strength, reduced MMA level and low sorption, it should have enough bulk. On the other hand, for reduced solubility, a thinner base is recommended.
- ✓ Pre-soaking of dentures in warm water for seven days after deflasking, before polishing, does not appear to have a deleterious effect on the strength of the dentures.

- ✓ A denture should be polished after immersion in warm water since polishing reduces solubility and would decrease leaching of monomer intra-orally and during overnight water storage.
- ✓ It is important to polish a denture base after water immersion following manufacturing and after chair-side adjustments to limit solubility.
- ✓ Material leaching from denture bases has been associated with reduced cell viability. This effect is unpredictable and needs further investigation.
- ✓ Since polishing is associated with lower solubility, it may be recommended that dentures be polished on the fitting surface as well for patients with known allergic reactions to denture-base resins.



### 4.7. Conclusion

Within the limitations of this *in vitro* study and the material used, the following conclusions may be drawn:

- Polishing of the tested denture base material will significantly lower solubility, but not the sorption. Degradation of the material due to sorption is not expected to be reduced by polishing.
- Within limits, it is acceptable to alter the mixing ratios without adversely affecting sorption or solubility.
- Since polishing reduces the solubility of a denture base acrylic, pre-soaking should be done after polymerization, but before polishing.

- An inverse proportional relationship exists between sorption and solubility with higher soaking temperatures leading to a lower sorption and higher solubility.
- An inverted relationship between sorption and solubility, as it relates to specimen thickness, was found.
- Different water immersion temperatures did not have an influence on the denture base strength.
- Short- and long-term exposures to eluates of a PMMA have a negative effect on cell viability. For water-stored and polished disks, this effect is time-dependent, with a higher viability for 48 hour- than for 24 hour-eluates.
- The cytotoxic potential of PMMA-eluates appears to fluctuate over time.



**4.8. Future research**

The results of this study in part and in general, can be used for future research. Future research could investigate other factors affecting the sorption and solubility of a type 1, class 1 denture base material and compare the sorption and solubility findings with cytotoxicity results by means of *in vitro* cytotoxicity testing.

The combination of pre-leaching and polishing could be included as a test group.

In several other cytotoxicity studies, the MTT-assay and the $^{3}$H-thymidine incorporation were used together, the MTT tested negative or unaffected, but, positive with the incorporation of $^{3}$H-thymidine , revealing more accuracy with the use of $^{3}$H-thymidine incorporation.

By means of high performance liquid chromatography (HPLC), reversed-phase high- performance liquid chromatography (HPLC) and gas-chromatographic (GC) methods, the chemical components of the eluate and the concentration of MMA monomer can be identified.

The use of thermo-cycling tests can be used to investigate its influence on the sorption and solubility factors of this study in future investigations.

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***APPENDIX A : Datasheet of raw data for polishing**

| | Specimen | Day 1 | Day 4 | Day 6 | Day 7 | Day 8 | Day 10 | Day 11 | Day 12 | Day 13 | Day 15 | M1 | M2 | Day 24 | Day 27 | Day 28 | Day 30 | Day 31 | Day 32 | M3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Polished | 5.4119 | 5.3959 | 5.3914 | 5.3903 | 5.3898 | 5.2862 | 5.2859 | 5.2856 | 5.2852 | 5.2855 | | 5.3837 | 5.3290 | 5.2846 | 5.2831 | 5.2821 | 5.2812 | 5.2812 | 5.2812 |
| 2 | Polished | 5.2106 | 5.1965 | 5.1934 | 5.1929 | 5.1925 | 5.1934 | 5.1921 | 5.1924 | 5.1923 | 5.1925 | 5.1923 | 5.2850 | 5.2320 | 5.1910 | 5.1900 | 5.1889 | 5.1887 | 5.1889 | 5.1887 |
| 3 | Polished | 5.0508 | 5.0368 | 5.0331 | 5.0328 | 5.0318 | 5.0328 | 5.0316 | 5.0321 | 5.0320 | 5.0319 | 5.0320 | 5.1231 | 5.0705 | 5.0284 | 5.0275 | 5.0268 | 5.0261 | 5.0260 | 5.0260 |
| 4 | Polished | 4.7666 | 4.7565 | 4.7541 | 4.7536 | 4.7530 | 4.5906 | 4.5901 | 4.5897 | 4.5894 | 4.5891 | | 4.6680 | 4.6134 | 4.5816 | 4.5815 | 4.5816 | 4.5812 | 4.5811 | 4.5815 |
| 5 | Polished | 5.2075 | 5.1886 | 5.1836 | 5.1825 | 5.1815 | 5.0765 | 5.0760 | 5.0759 | 5.0757 | 5.0758 | 5.0759 | 5.1678 | 5.1197 | 5.0770 | 5.0754 | 5.0733 | 5.0736 | 5.0732 | 5.0732 |
| 6 | Polished | 4.9386 | 4.9217 | 4.9178 | 4.9169 | 4.9161 | 4.9170 | 4.9187 | 4.9165 | 4.9160 | 4.9159 | 4.9159 | 5.0058 | 4.9539 | 4.9134 | 4.9124 | 4.9111 | 4.9109 | 4.9114 | 4.9109 |
| 7 | Polished | 5.0324 | 5.0157 | 5.0122 | 5.0112 | 5.0105 | 5.0116 | 5.0103 | 5.0107 | 5.0102 | 5.0104 | 5.0104 | 5.1005 | 5.0458 | 5.0059 | 5.0048 | 5.0035 | 5.0046 | 5.0044 | 5.0044 |
| 8 | Polished | 5.1235 | 5.1034 | 5.0989 | 5.0977 | 5.0969 | 5.0044 | 5.0045 | 5.0038 | 5.0036 | 5.0038 | 5.0036 | 5.0951 | 5.0435 | 5.0027 | 5.0013 | 4.9999 | 4.9996 | 4.9999 | 4.9999 |
| 9 | Polished | 5.0116 | 4.9980 | 4.9944 | 4.9936 | 4.9934 | 4.9941 | 4.9930 | 4.9930 | 4.9931 | 4.9931 | 4.9930 | 5.0863 | 5.0298 | 4.9892 | 4.9881 | 4.9872 | 4.9866 | 4.9872 | 4.9872 |
| 10 | Polished | 5.2368 | 5.2213 | 5.2170 | 5.2161 | 5.2157 | 5.1064 | 5.1060 | 5.1063 | 5.1058 | 5.1058 | 5.1058 | 5.1995 | 5.1495 | 5.1071 | 5.1055 | 5.1039 | 5.1032 | 5.1037 | 5.1037 |
| 11 | Polished | 4.9218 | 4.9090 | 4.9058 | 4.9055 | 4.9054 | 4.9060 | 4.9050 | 4.9052 | 4.9052 | 4.9053 | 4.9054 | 4.9953 | 4.9436 | 4.9038 | 4.9029 | 4.9019 | 4.9023 | 4.9022 | 4.9022 |
| 12 | Polished | 5.7626 | 5.7455 | 5.7404 | 5.7393 | 5.7389 | 5.6200 | 5.6200 | 5.6194 | 5.6190 | 5.6190 | 5.6190 | 5.7208 | 5.6660 | 5.6205 | 5.6187 | 5.6166 | 5.6162 | 5.6164 | 5.6164 |
| 13 | Unpolished | 5.1424 | 5.1240 | 5.1191 | 5.1183 | 5.1179 | 5.1183 | 5.1176 | 5.1176 | 5.1173 | 5.1172 | 5.1176 | 5.2091 | 5.1603 | 5.1180 | 5.1164 | 5.1149 | 5.1149 | 5.1147 | 5.1149 |
| 14 | Unpolished | 4.7868 | 4.7750 | 4.7724 | 4.7718 | 4.7717 | 4.6882 | 4.6884 | 4.6879 | 4.6876 | 4.6877 | 4.6877 | 4.7759 | 4.7229 | 4.6860 | 4.6853 | 4.6843 | 4.6849 | 4.6844 | 4.6844 |
| 15 | Unpolished | 4.9958 | 4.9761 | 4.9715 | 4.9708 | 4.9704 | 4.9710 | 4.9698 | 4.9703 | 4.9699 | 4.9700 | 4.9700 | 5.0622 | 5.0122 | 4.9696 | 4.9681 | 4.9675 | 4.9665 | 4.9664 | 4.9664 |
| 16 | Unpolished | 4.7583 | 4.7470 | 4.7439 | 4.7432 | 4.7430 | 4.7443 | 4.7439 | 4.7438 | 4.7433 | 4.7433 | 4.7430 | 4.8294 | 4.7776 | 4.7393 | 4.7387 | 4.7385 | 4.7379 | 4.7381 | 4.7381 |
| 17 | Unpolished | 4.8425 | 4.8315 | 4.8282 | 4.8276 | 4.8274 | 4.8285 | 4.8280 | 4.8277 | 4.8274 | 4.8273 | 4.8274 | 4.9102 | 4.8579 | 4.8212 | 4.8202 | 4.8206 | 4.8196 | 4.8198 | 4.8198 |
| 18 | Unpolished | 5.5124 | 5.4927 | 5.4864 | 5.4852 | 5.4844 | 5.3750 | 5.3751 | 5.3745 | 5.3741 | 5.3742 | 5.3742 | 5.4704 | 5.4186 | 5.3750 | 5.3734 | 5.3714 | 5.3707 | 5.3710 | |
| 19 | Unpolished | 5.0717 | 5.0579 | 5.0540 | 5.0534 | 5.0529 | 4.9447 | 4.9444 | 4.9442 | 4.9440 | 4.9442 | 4.9442 | 5.0399 | 4.9852 | 4.9434 | 4.9422 | 4.9412 | 4.9413 | 4.9414 | 4.9413 |
| 20 | Unpolished | 5.0763 | 5.0579 | 5.0525 | 5.0520 | 5.0507 | 4.9425 | 4.9421 | 4.9421 | 4.9420 | 4.9424 | 4.9421 | 5.0354 | 4.9863 | 4.9439 | 4.9424 | 4.9407 | 4.9409 | 4.9407 | 4.9409 |
| 21 | Unpolished | 4.9713 | 4.9539 | 4.9488 | 4.9481 | 4.9473 | 4.9484 | 4.9479 | 4.9478 | 4.9475 | 4.9475 | 4.9478 | 5.0388 | 4.9896 | 4.9472 | 4.9457 | 4.9450 | 4.9442 | 4.9443 | 4.9443 |
| 22 | Unpolished | 5.0467 | 5.0291 | 5.0240 | 5.0234 | 5.0226 | 5.0237 | 5.0231 | 5.0227 | 5.0225 | 5.0225 | 5.0225 | 5.1114 | 5.0636 | 5.0209 | 5.0198 | 5.0189 | 5.0182 | 5.0181 | 5.0181 |
| 23 | Unpolished | 5.2393 | 5.2282 | 5.2243 | 5.2240 | 5.2234 | 5.0974 | 5.0972 | 5.0971 | 5.0971 | 5.0971 | 5.0972 | 5.1906 | 5.1407 | 5.0986 | 5.0973 | 5.0956 | 5.0958 | 5.0956 | 5.0958 |
| 24 | Unpolished | 4.8906 | 4.8773 | 4.8745 | 4.8736 | 4.8730 | 4.7598 | 4.7595 | 4.7598 | 4.7590 | 4.7599 | 4.7598 | 4.8482 | 4.7972 | 4.7572 | 4.7562 | 4.7548 | 4.7554 | 4.7554 | 4.7554 |



* Some days were not included to allow the fit of relevant data on one datasheet for ease of reading.

***APPENDIX B: Datasheet of raw data for mixing ratios**

| *Specimen* | | *Day 1* | *Day 6* | *Day 7* | *Day 8* | *Day 9* | *Day 10* | *Day 11* | *Day 12* | *Day 13* | *Day 14* | *Day15* | *M1* | *M2* | *Day 38* | *Day 39* | *Day 40* | *Day 41* | *Day 42* | *M3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| L1 | Liquid | 2.7774 | 2.7171 | 2.7159 | 2.7150 | 2.7144 | 2.7136 | 2.7126 | 2.7125 | 2.7114 | 2.7108 | 2.7102 | 2.7125 | 2.7509 | 2.7509 | 2.7000 | 2.6990 | 2.6988 | 2.6986 | 2.6986 |
| L2 | Liquid | 3.2167 | 3.1498 | 3.1492 | 3.1490 | 3.1489 | 3.1485 | 3.1478 | 3.1482 | 3.1476 | 3.1478 | 3.1470 | 3.1490 | 3.2073 | 3.2073 | 3.1432 | 3.1420 | 3.1418 | 3.1418 | 3.1418 |
| L3 | Liquid | 2.8894 | 2.8292 | 2.8289 | 2.8291 | 2.8285 | 2.8288 | 2.8283 | 2.8285 | 2.8278 | 2.8278 | 2.8276 | 2.8291 | 2.8868 | 2.8868 | 2.8246 | 2.8237 | 2.8236 | 2.8236 | 2.8236 |
| L4 | Liquid | 2.7563 | 2.7002 | 2.6999 | 2.6998 | 2.6995 | 2.6997 | 2.6991 | 2.6995 | 2.6991 | 2.6988 | 2.6987 | 2.6998 | 2.7534 | 2.7534 | 2.6966 | 2.6956 | 2.6955 | 2.6955 | 2.6955 |
| L5 | Liquid | 3.3640 | 3.2967 | 3.2962 | 3.2965 | 3.2961 | 3.2962 | 3.2956 | 3.2958 | 3.2952 | 3.2951 | 3.2952 | 3.2962 | 3.3559 | 3.3559 | 3.2911 | 3.2901 | 3.2901 | 3.2899 | 3.2899 |
| L6 | Liquid | 2.9220 | 2.8604 | 2.8599 | 2.8601 | 2.8598 | 2.8595 | 2.8590 | 2.8591 | 2.8583 | 2.8582 | 2.8580 | 2.8601 | 2.9134 | 2.9134 | 2.8542 | 2.8531 | 2.8531 | 2.8531 | 2.8531 |
| L7 | Liquid | 3.1428 | 3.0776 | 3.0770 | 3.0772 | 3.0770 | 3.0768 | 3.0765 | 3.0767 | 3.0760 | 3.0759 | 3.0757 | 3.0772 | 3.1340 | 3.1340 | 3.0727 | 3.0717 | 3.0714 | 3.0715 | 3.0715 |
| L8 | Liquid | 3.2792 | 3.2133 | 3.2131 | 3.2129 | 3.2129 | 3.2127 | 3.2123 | 3.2127 | 3.2123 | 3.2119 | 3.2119 | 3.2131 | 3.2771 | 3.2771 | 3.2099 | 3.2088 | 3.2087 | 3.2086 | 3.2087 |
| L9 | Liquid | 3.0427 | 2.9813 | 2.9809 | 2.9814 | 2.9810 | 2.9812 | 2.9809 | 2.9812 | 2.9807 | 2.9807 | 2.9806 | 2.9812 | 3.0434 | 3.0434 | 2.9797 | 2.9784 | 2.9788 | 2.9789 | 2.9789 |
| L10 | Liquid | 2.9419 | 2.8870 | 2.8867 | 2.8870 | 2.8867 | 2.8868 | 2.8864 | 2.8867 | 2.8861 | 2.8860 | 2.8857 | 2.8868 | 2.9403 | 2.9403 | 2.8830 | 2.8823 | 2.8821 | 2.8821 | 2.8821 |
| L11 | Liquid | 3.1930 | 3.1286 | 3.1280 | 3.1281 | 3.1278 | 3.1281 | 3.1278 | 3.1280 | 3.1276 | 3.1275 | 3.1274 | 3.1281 | 3.1946 | 3.1946 | 3.1262 | 3.1250 | 3.1249 | 3.1247 | 3.1249 |
| L12 | Liquid | 2.7205 | 2.6658 | 2.6655 | 2.6655 | 2.6655 | 2.6655 | 2.6650 | 2.6654 | 2.6649 | 2.6645 | 2.6646 | 2.6658 | 2.7183 | 2.7183 | 2.6626 | 2.6616 | 2.6617 | 2.6616 | 2.6617 |
| | | | | | | | | | | | | | | | | | | | | |
| C1 | Control | 3.4450 | 3.3770 | 3.3759 | 3.3748 | 3.3738 | 3.3729 | 3.3718 | 3.3712 | 3.3704 | 3.3698 | 3.3691 | | 3.4145 | 3.4145 | 3.3577 | 3.3559 | 3.3558 | 3.3559 | 3.3558 |
| C2 | Control | 3.1816 | 3.1188 | 3.1180 | 3.1171 | 3.1160 | 3.1152 | 3.1136 | 3.1131 | 3.1119 | 3.1110 | 3.1100 | | 3.1511 | 3.1511 | 3.0984 | 3.0967 | 3.0964 | 3.0966 | 3.0966 |
| C3 | Control | 3.1726 | 3.1132 | 3.1131 | 3.1133 | 3.1132 | 3.1132 | 3.1130 | 3.1132 | 3.1131 | 3.1131 | 3.1130 | 3.1131 | 3.1811 | 3.1811 | 3.1130 | 3.1111 | 3.1111 | 3.1113 | 3.1111 |
| C4 | Control | 3.4195 | 3.3559 | 3.3549 | 3.3542 | 3.3535 | 3.3528 | 3.3521 | 3.3510 | 3.3499 | 3.3488 | 3.3481 | | 3.4004 | 3.4004 | 3.3380 | 3.3363 | 3.1111 | 3.3365 | 3.1111 |
| C5 | Control | 3.3691 | 3.3039 | 3.3025 | 3.3011 | 3.2999 | 3.2999 | 3.2980 | 3.2970 | 3.2962 | 3.2954 | 3.2949 | 3.2999 | 3.3377 | 3.3377 | 3.2818 | 3.2803 | 3.2800 | 3.2800 | 3.28 |
| C6 | Control | 3.0302 | 2.9760 | 2.9758 | 2.9758 | 2.9757 | 2.9756 | 2.9755 | 2.9759 | 2.9752 | 2.9751 | 2.9754 | 2.9758 | 3.0337 | 3.0337 | 2.9731 | 2.9714 | 2.9715 | 2.9715 | 2.9715 |
| C7 | Control | 3.0903 | 3.0333 | 3.0332 | 3.0334 | 3.0333 | 3.0333 | 3.0332 | 3.0333 | 3.0333 | 3.0332 | 3.0333 | 3.0332 | 3.0960 | 3.0960 | 3.0327 | 3.0311 | 3.0311 | 3.0310 | 3.031 |
| C8 | Control | 3.4830 | 3.4212 | 3.4210 | 3.4206 | 3.4200 | 3.4197 | 3.4191 | 3.4191 | 3.4188 | 3.4184 | 3.4181 | 3.4210 | 3.4716 | 3.4716 | 3.4125 | 3.4109 | 3.4108 | 3.4108 | 3.4108 |
| C9 | Control | 3.0932 | 3.0376 | 3.0373 | 3.0375 | 3.0373 | 3.0373 | 3.0372 | 3.0373 | 3.0371 | 3.0369 | 3.0372 | 3.0373 | 3.0990 | 3.0990 | 3.0360 | 3.0344 | 3.0344 | 3.0343 | 3.0343 |
| C10 | Control | 3.1825 | 3.1252 | 3.1247 | 3.1248 | 3.1246 | 3.1244 | 3.1246 | 3.1242 | 3.1242 | 3.1242 | 3.1243 | 3.1252 | 3.1882 | 3.1882 | 3.1226 | 3.1209 | 3.1211 | 3.1209 | 3.1211 |
| C11 | Control | 2.8295 | 2.7760 | 2.7752 | 2.7745 | 2.7737 | 2.7730 | 2.7724 | 2.7721 | 2.7713 | 2.7711 | 2.7706 | 2.7706 | 2.8112 | 2.8112 | 2.7619 | 2.7603 | 2.7605 | 2.7601 | 2.7605 |
| C12 | Control | 3.5250 | 3.4636 | 3.4630 | 3.4631 | 3.4630 | 3.4628 | 3.4630 | 3.4627 | 3.4627 | 3.4628 | 3.4630 | 3.4631 | 3.5304 | 3.5304 | 3.4621 | 3.4601 | 3.4603 | 3.4601 | 3.4603 |
| | | | | | | | | | | | | | | | | | | | | |
| P1 | Powder | 3.1823 | 3.1197 | 3.1192 | 3.1185 | 3.1181 | 3.1178 | 3.1176 | 3.1171 | 3.1168 | 3.1166 | 3.1162 | 3.1176 | 3.1615 | 3.1615 | 3.1096 | 3.1088 | 3.1091 | 3.1089 | 3.1089 |
| P2 | Powder | 3.7957 | 3.7260 | 3.7254 | 3.7248 | 3.7245 | 3.7243 | 3.7244 | 3.7241 | 3.7240 | 3.7238 | 3.7237 | 3.7243 | 3.7947 | 3.7947 | 3.7210 | 3.7196 | 3.7198 | 3.7195 | 3.7198 |
| P3 | Powder | 3.8801 | 3.8045 | 3.8036 | 3.8026 | 3.8020 | 3.8010 | 3.8009 | 3.8004 | 3.7997 | 3.7990 | 3.7988 | 3.8009 | 3.8508 | 3.8508 | 3.7898 | 3.7889 | 3.7893 | 3.7889 | |
| P4 | Powder | 3.9233 | 3.8544 | 3.8534 | 3.8524 | 3.8521 | 3.8521 | 3.8520 | 3.8520 | 3.8520 | 3.8521 | 3.8518 | 3.8521 | 3.9292 | 3.9292 | 3.8532 | 3.8505 | 3.8507 | 3.8507 | 3.8507 |
| P5 | Powder | 4.7843 | 4.7150 | 4.7130 | 4.7114 | 4.7105 | 4.7102 | 4.7098 | 4.7096 | 4.7094 | 4.7095 | 4.7090 | 4.7096 | 4.7917 | 4.7917 | 4.7104 | 4.7058 | 4.7057 | 4.7057 | 4.7057 |
| P6 | Powder | 5.2373 | 5.1672 | 5.1649 | 5.1631 | 5.1622 | 5.1618 | 5.1615 | 5.1616 | 5.1612 | 5.1610 | 5.1609 | 5.1616 | 5.2473 | 5.2473 | 5.1654 | 5.1602 | 5.1599 | 5.1592 | |
| P7 | Powder | 4.1570 | 4.0861 | 4.0849 | 4.0842 | 4.0838 | 4.0835 | 4.0837 | 4.0831 | 4.0833 | 4.0829 | 4.0830 | 4.0837 | 4.1607 | 4.1607 | 4.0830 | 4.0801 | 4.0806 | 4.0803 | |
| P8 | Powder | 4.6935 | 4.6221 | 4.6208 | 4.6197 | 4.6193 | 4.6193 | 4.6192 | 4.6188 | 4.6188 | 4.6184 | 4.6186 | 4.6193 | 4.6972 | 4.6972 | 4.6182 | 4.6152 | 4.6154 | 4.6150 | 4.6154 |
| P9 | Powder | 3.9980 | 3.9274 | 3.9270 | 3.9267 | 3.9263 | 3.9262 | 3.9262 | 3.9260 | 3.9258 | 3.9256 | 3.9255 | 3.9262 | 3.9918 | 3.9918 | 3.9220 | 3.9207 | 3.9211 | 3.9211 | 3.9211 |
| P10 | Powder | 3.5568 | 3.4889 | 3.4883 | 3.4879 | 3.4874 | 3.4872 | 3.4873 | 3.4867 | 3.4866 | 3.4862 | 3.4861 | 3.4872 | 3.5452 | 3.5452 | 3.4810 | 3.4800 | 3.4804 | 3.4804 | 3.4804 |
| P11 | Powder | 4.3817 | 4.3123 | 4.3112 | 4.3109 | 4.3103 | 4.3102 | 4.3105 | 4.3099 | 4.3101 | 4.3098 | 4.3099 | 4.3102 | 4.3850 | 4.3850 | 4.3091 | 4.3068 | 4.3072 | 4.3071 | 4.3071 |
| P12 | Powder | 3.8778 | 3.8093 | 3.8088 | 3.8085 | 3.8083 | 3.8083 | 3.8083 | 3.8082 | 3.8081 | 3.8081 | 3.8079 | 3.8083 | 3.8761 | 3.8761 | 3.8063 | 3.8049 | 3.8052 | 3.8056 | |

*Some days were not included to allow the fit of relevant data on one datasheet for ease of reading.

**APPENDIX C: Datasheet of raw dara for water imersion temperatures**

| *Temperature (ºC)* | *Specimen* | *Day 1* | *Day 4* | *Day 5* | *Day 6* | *Day 7* | *Day 8* | *M1* | *M2* | *Day 16* | *Day 17* | *Day 18* | *Day 19* | *Day 20* | *M3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 22 | 3 | 1.6764 | 1.672 | 1.6719 | 1.6719 | 1.672 | 1.6718 | 1.6719 | 1.7167 | 1.6776 | 1.6731 | 1.6722 | 1.6721 | 1.6721 | 1.6721 |
| 22 | 7 | 1.6793 | 1.6753 | 1.6753 | 1.6755 | 1.6753 | 1.6752 | 1.6753 | 1.7275 | 1.6803 | 1.6761 | 1.6752 | 1.6752 | 1.6752 | 1.6752 |
| 22 | 11 | 1.9951 | 1.9866 | 1.9865 | 1.9864 | 1.9865 | 1.9863 | 1.9865 | 2.0461 | 1.9968 | 1.9887 | 1.9871 | 1.9867 | 1.9867 | 1.9867 |
| 22 | 15 | 1.888 | 1.8812 | 1.8812 | 1.881 | 1.881 | 1.8808 | 1.8812 | 1.9383 | 1.8897 | 1.8828 | 1.8813 | 1.881 | 1.8811 | 1.8811 |
| 22 | 19 | 1.892 | 1.8855 | 1.8853 | 1.8853 | 1.8855 | 1.8853 | 1.8853 | 1.9395 | 1.8934 | 1.887 | 1.8854 | 1.8854 | 1.8852 | 1.8854 |
| 22 | 23 | 1.8696 | 1.8621 | 1.8617 | 1.8617 | 1.8618 | 1.8616 | 1.8617 | 1.9119 | 1.8701 | 1.8634 | 1.862 | 1.8618 | 1.8618 | 1.8618 |
| 22 | 27 | 1.8351 | 1.8278 | 1.828 | 1.82745 | 1.8278 | 1.8277 | 1.828 | 1.8801 | 1.8354 | 1.8294 | 1.828 | 1.828 | 1.8279 | 1.828 |
| 22 | 31 | 1.9712 | 1.9624 | 1.9622 | 1.9619 | 1.962 | 1.9618 | 1.9622 | 2.0183 | 1.9711 | 1.9642 | 1.9622 | 1.9622 | 1.9621 | 1.9622 |
| 22 | 35 | 2.0353 | 2.0245 | 2.0243 | 2.024 | 2.0243 | 2.024 | 2.0243 | 2.0797 | 2.0349 | 2.0269 | 2.0248 | 2.0245 | 2.0245 | 2.0245 |
| 22 | 39 | 2.052 | 2.0419 | 2.0418 | 2.0416 | 2.0418 | 2.0415 | 2.0418 | 2.0969 | 2.0514 | 2.0438 | 2.042 | 2.0418 | 2.0419 | 2.0418 |
| 22 | 43 | 2.269 | 2.2552 | 2.2549 | 2.255 | 2.2551 | 2.2547 | 2.255 | 2.3148 | 2.2688 | 2.2587 | 2.2558 | 2.2552 | 2.255 | 2.255 |
| 22 | 46 | 1.8285 | 1.8221 | 1.8218 | 1.8216 | 1.8221 | 1.8219 | 1.8216 | 1.8726 | 1.8291 | 1.8229 | 1.8217 | 1.8215 | 1.8215 | 1.8215 |
| 22 | 50 | 2.0615 | 2.0513 | 2.0511 | 2.0505 | 2.0507 | 2.0509 | 2.0511 | 2.1087 | 2.0614 | 2.0532 | 2.0512 | 2.0509 | 2.0508 | 2.0508 |
| 22 | 54 | 2.0857 | 2.0758 | 2.0759 | 2.0757 | 2.0757 | 2.0756 | 2.0759 | 2.1297 | 2.0862 | 2.0778 | 2.0759 | 2.0757 | 2.0756 | 2.0757 |
| | | | | | | | | | | | | | | | |
| 37 | 1 | 1.6527 | 1.6489 | 1.6486 | 1.6486 | 1.6487 | 1.6485 | 1.6486 | 1.704 | 1.6546 | 1.649 | 1.6482 | 1.6481 | 1.648 | 1.6481 |
| 37 | 4 | 1.6234 | 1.6197 | 1.6196 | 1.6198 | 1.6196 | 1.6198 | 1.6196 | 1.6651 | 1.6254 | 1.62 | 1.6194 | 1.6191 | 1.619 | 1.619 |
| 37 | 8 | 1.6552 | 1.6508 | 1.6506 | 1.6506 | 1.6506 | 1.6505 | 1.6506 | 1.6953 | 1.6569 | 1.6512 | 1.6499 | 1.6498 | 1.6499 | 1.6498 |
| 37 | 12 | 1.7601 | 1.755 | 1.7551 | 1.755 | 1.7551 | 1.7549 | 1.7551 | 1.8048 | 1.7622 | 1.7556 | 1.7544 | 1.7543 | 1.7543 | 1.7543 |
| 37 | 18 | 2.1205 | 2.1111 | 2.1111 | 2.1109 | 2.111 | 2.1109 | 2.1111 | 2.1661 | 2.123 | 2.1126 | 2.1104 | 2.1096 | 2.1096 | 2.1096 |
| 37 | 22 | 1.7408 | 1.7363 | 1.7359 | 1.7359 | 1.736 | 1.7359 | 1.7359 | 1.7798 | 1.7433 | 1.7369 | 1.7358 | 1.7356 | 1.7354 | 1.7356 |
| 37 | 26 | 1.7243 | 1.7195 | 1.7196 | 1.7191 | 1.7193 | 1.7191 | 1.7196 | 1.7655 | 1.7258 | 1.7199 | 1.7188 | 1.7185 | 1.7186 | 1.7186 |
| 37 | 30 | 1.858 | 1.8517 | 1.8517 | 1.8514 | 1.8514 | 1.8513 | 1.8517 | 1.8976 | 1.86 | 1.8524 | 1.851 | 1.8507 | 1.8507 | 1.8507 |
| 37 | 34 | 2.0761 | 2.0652 | 2.0652 | 2.0649 | 2.0652 | 2.0649 | 2.0652 | 2.1188 | 2.0777 | 2.0675 | 2.0652 | 2.0647 | 2.0646 | 2.0646 |
| 37 | 38 | 1.8218 | 1.8152 | 1.8151 | 1.8151 | 1.8152 | 1.815 | 1.8151 | 1.8598 | 1.8232 | 1.8154 | 1.8142 | 1.8142 | 1.8142 | 1.8142 |
| 37 | 42 | 2.0571 | 2.0464 | 2.0463 | 2.0462 | 2.0463 | 2.046 | 2.0463 | 2.104 | 2.0585 | 2.0482 | 2.0457 | 2.0452 | 2.0451 | 2.0451 |
| 37 | 47 | 1.8422 | 1.836 | 1.8356 | 1.8353 | 1.8355 | 1.8355 | 1.8355 | 1.8827 | 1.8438 | 1.8364 | 1.8348 | 1.8346 | 1.8346 | 1.8346 |
| 37 | 51 | 2.0946 | 2.0841 | 2.0842 | 2.0839 | 2.0841 | 2.0839 | 2.0842 | 2.1537 | 2.0964 | 2.086 | 2.0837 | 2.0832 | 2.0832 | 2.0832 |
| 37 | 55 | 1.6167 | 1.5996 | 1.5997 | 1.5995 | 1.5995 | 1.5994 | 1.5997 | 1.6408 | 1.6042 | 1.5993 | 1.5984 | 1.5983 | 1.5984 | 1.5983 |
| | | | | | | | | | | | | | | | |
| 55 | 5 | 1.6601 | 1.6562 | 1.6564 | 1.6563 | 1.6564 | 1.6562 | 1.6564 | 1.6927 | 1.6601 | 1.6557 | 1.6548 | 1.6545 | 1.6546 | 1.6546 |
| 55 | 9 | 1.5767 | 1.5733 | 1.5733 | 1.5733 | 1.5733 | 1.573 | 1.5733 | 1.6062 | 1.5752 | 1.5712 | 1.5705 | 1.5702 | 1.5702 | 1.5702 |
| 55 | 13 | 1.9582 | 1.95 | 1.95 | 1.95 | 1.9501 | 1.9499 | 1.95 | 1.9973 | 1.9591 | 1.9512 | 1.9494 | 1.949 | 1.949 | 1.949 |
| 55 | 17 | 1.9338 | 1.9266 | 1.9264 | 1.9263 | 1.9264 | 1.9263 | 1.9264 | 1.9731 | 1.9353 | 1.9274 | 1.9257 | 1.9253 | 1.9254 | 1.9254 |
| 55 | 21 | 1.901 | 1.8944 | 1.8942 | 1.8942 | 1.8944 | 1.8941 | 1.8942 | 1.9378 | 1.9015 | 1.8942 | 1.8928 | 1.8924 | 1.8924 | 1.8924 |
| 55 | 25 | 1.9312 | 1.9235 | 1.9233 | 1.9232 | 1.9233 | 1.9231 | 1.9233 | 1.9715 | 1.9313 | 1.9239 | 1.9224 | 1.9221 | 1.9221 | 1.9221 |
| 55 | 32 | 1.7933 | 1.7869 | 1.7864 | 1.7866 | 1.7868 | 1.7864 | 1.7866 | 1.8315 | 1.7933 | 1.7874 | 1.786 | 1.7857 | 1.7858 | 1.7858 |
| 55 | 33 | 1.7836 | 1.7771 | 1.7768 | 1.7767 | 1.7769 | 1.7768 | 1.7767 | 1.8179 | 1.7834 | 1.7774 | 1.7764 | 1.7762 | 1.7762 | 1.7762 |
| 55 | 36 | 1.8543 | 1.8469 | 1.8468 | 1.8464 | 1.8469 | 1.8465 | 1.8468 | 1.8911 | 1.854 | 1.8474 | 1.846 | 1.8458 | 1.8459 | 1.8458 |
| 55 | 37 | 1.9753 | 1.9659 | 1.9658 | 1.9659 | 1.9659 | 1.9657 | 1.9658 | 2.0154 | 1.9747 | 1.9669 | 1.965 | 1.9648 | 1.9647 | 1.9648 |
| 55 | 41 | 1.9432 | 1.9344 | 1.9342 | 1.9342 | 1.9343 | 1.934 | 1.9342 | 1.9789 | 1.942 | 1.9344 | 1.9326 | 1.9324 | 1.9323 | 1.9324 |
| 55 | 45 | 1.7674 | 1.7612 | 1.7615 | 1.7612 | 1.7613 | 1.7609 | 1.7613 | 1.8078 | 1.7669 | 1.761 | 1.7601 | 1.7599 | 1.7601 | 1.7599 |
| 55 | 48 | 2.0553 | 2.0451 | 2.045 | 2.0445 | 2.0447 | 2.0446 | 2.045 | 2.0945 | - | 2.0454 | 2.0435 | 2.0431 | 2.0432 | 2.0434 |
| 55 | 52 | 1.8541 | 1.8474 | 1.8475 | 1.8472 | 1.8473 | 1.8471 | 1.8475 | 1.8893 | 1.8539 | 1.8473 | 1.8461 | 1.8462 | 1.8464 | 1.8462 |
| | | | | | | | | | | | | | | | |
| 70 | 2 | 1.6914 | 1.6868 | 1.6868 | 1.6868 | 1.6868 | 1.6866 | 1.6868 | 1.724 | 1.6909 | 1.6863 | 1.6852 | 1.6851 | 1.6853 | 1.6851 |
| 70 | 6 | 1.6825 | 1.6787 | 1.6788 | 1.6787 | 1.6786 | 1.6785 | 1.6788 | 1.7114 | 1.6787 | 1.6749 | 1.6739 | 1.6736 | 1.6738 | 1.6738 |
| 70 | 10 | 2.0513 | 2.0414 | 2.0416 | 2.0415 | 2.0416 | 2.0414 | 2.0416 | 2.0872 | 2.0515 | 2.0433 | 2.0412 | 2.0408 | 2.041 | 2.041 |
| 70 | 14 | 2.0714 | 2.0622 | 2.0622 | 2.062 | 2.0622 | 2.062 | 2.0622 | 2.1047 | 2.0695 | 2.061 | 2.0593 | 2.0588 | 2.0589 | 2.0589 |
| 70 | 16 | 1.7357 | 1.7309 | 1.7309 | 1.731 | 1.731 | 1.7307 | 1.7309 | 1.771 | 1.7366 | 1.7312 | 1.73 | 1.73 | 1.7302 | 1.73 |
| 70 | 20 | 1.7587 | 1.7539 | 1.7539 | 1.7537 | 1.754 | 1.7537 | 1.7539 | 1.7925 | 1.7595 | 1.7539 | 1.7527 | 1.7527 | 1.7529 | 1.7527 |
| 70 | 24 | 1.9344 | 1.9265 | 1.9263 | 1.9261 | 1.9262 | 1.9261 | 1.9263 | 1.9714 | 1.9336 | 1.9263 | 1.9244 | 1.9244 | 1.9245 | 1.9244 |
| 70 | 28 | 1.6919 | 1.6872 | 1.6872 | 1.6867 | 1.6871 | 1.6868 | 1.6872 | 1.7237 | 1.691 | 1.6859 | 1.6847 | 1.6848 | 1.685 | 1.6848 |
| 70 | 29 | 1.814 | 1.8072 | 1.8071 | 1.807 | 1.807 | 1.8068 | 1.8071 | 1.8461 | 1.8126 | 1.8062 | 1.8049 | 1.8048 | 1.8049 | 1.8048 |
| 70 | 40 | 2.2311 | 2.2183 | 2.218 | 2.2179 | 2.218 | 2.2178 | 2.2179 | 2.2647 | 2.2264 | 2.216 | 2.2135 | 2.2131 | 2.2131 | 2.2131 |
| 70 | 44 | 1.5547 | 1.5513 | 1.5516 | 1.5512 | 1.5511 | 1.5508 | 1.5511 | 1.5976 | 1.5528 | 1.5493 | | 1.5485 | 1.5487 | 1.5487 |
| 70 | 49 | 2.0877 | 2.0776 | 2.0776 | 2.077 | 2.0772 | 2.0774 | 2.0776 | 2.1222 | 2.0856 | 2.0767 | 2.0744 | 2.0745 | 2.0747 | 2.0745 |
| 70 | 53 | 1.8245 | 1.8185 | 1.8185 | 1.8181 | 1.8186 | 1.8182 | 1.8185 | 1.8576 | 1.8237 | 1.8173 | 1.8162 | 1.8161 | 1.8162 | 1.8161 |

## APPENDIX D: Datasheet of raw data for cytotoxicity at 24 and 48 hours

24H

| CONTROL GROUP | | | | | AVERAGE |
|---|---|---|---|---|---|
| C1 | 0.683 | 0.715 | 0.714 | | 0.704 |
| C2 | 0.688 | 0.636 | 0.637 | | 0.654 |
| C3 | 0.658 | 0.631 | 0.686 | | 0.658 |
| C4 | 0.594 | 0.639 | 0.655 | | 0.629 |
| C5 | 0.552 | 0.71 | 0.666 | | 0.643 |
| C6 | 0.672 | 0.701 | 0.552 | | 0.642 |
| C7 | 0.588 | 0.546 | 0.625 | | 0.586 |
| C8 | 0.549 | 0.626 | 0.552 | | 0.576 |
| C9 | 0.636 | 0.654 | 0.613 | | 0.634 |
| | | | | | |
| POLISHED GROUP | | | | | |
| P1 | 0.616 | 0.526 | 0.596 | | 0.579 |
| P2 | 0.597 | 0.592 | 0.558 | | 0.582 |
| P3 | 0.686 | 0.559 | 0.532 | | 0.592 |
| P4 | 0.569 | 0.612 | 0.619 | | 0.600 |
| P5 | 0.609 | 0.555 | 0.579 | | 0.581 |
| P6 | 0.587 | 0.588 | 0.549 | | 0.575 |
| P7 | 0.532 | 0.569 | 0.574 | | 0.558 |
| P8 | 0.577 | 0.545 | 0.626 | | 0.583 |
| P9 | 0.635 | 0.676 | 0.603 | | 0.638 |

| WATER TEMPERATURE GROUP | | | | | |
|---|---|---|---|---|---|
| W1 | 0.556 | 0.582 | 0.592 | | 0.577 |
| W2 | 0.587 | 0.624 | 0.67 | | 0.627 |
| W3 | 0.567 | 0.555 | 0.637 | | 0.586 |
| W4 | 0.611 | 0.598 | 0.62 | | 0.610 |
| W5 | 0.639 | 0.649 | 0.646 | | 0.645 |
| W6 | 0.634 | 0.587 | 0.529 | | 0.583 |
| W7 | 0.571 | 0.537 | 0.613 | | 0.574 |
| W8 | 0.629 | 0.589 | 0.578 | | 0.599 |
| W9 | 0.576 | 0.603 | 0.593 | | 0.591 |

48H

| CONTROL GROUP | | | | | AVERAGE |
|---|---|---|---|---|---|
| C1 | 0.609 | 0.634 | 0.644 | | 0.629 |
| C2 | 0.613 | 0.675 | 0.694 | | 0.661 |
| C3 | 0.708 | 0.682 | 0.654 | | 0.681 |
| C4 | 0.628 | 0.568 | 0.643 | | 0.613 |
| C5 | 0.706 | 0.684 | 0.654 | | 0.681 |
| C6 | 0.628 | 0.568 | 0.643 | | 0.613 |
| C7 | 0.706 | 0.654 | 0.693 | | 0.684 |
| C8 | 0.533 | 0.529 | 0.601 | | 0.554 |
| C9 | 0.606 | 0.632 | 0.674 | | 0.637 |
| | | | | | |
| POLISHED GROUP | | | | | |
| P1 | 0.667 | 0.664 | 0.623 | | 0.651 |
| P2 | 0.647 | 0.663 | 0.625 | | 0.645 |
| P3 | 0.654 | 0.634 | 0.623 | | 0.637 |
| P4 | 0.697 | 0.661 | 0.692 | | 0.683 |
| P5 | 0.679 | 0.725 | 0.693 | | 0.699 |
| P6 | 0.625 | 0.639 | 0.688 | | 0.651 |
| P7 | 0.614 | 0.64 | 0.692 | | 0.649 |
| P8 | 0.669 | 0.708 | 0.657 | | 0.678 |
| P9 | 0.613 | 0.698 | 0.651 | | 0.654 |

| WATER TEMPERATURE GROUP | | | | | |
|---|---|---|---|---|---|
| W1 | 0.628 | 0.622 | 0.704 | | 0.651 |
| W2 | 0.548 | 0.563 | 0.661 | | 0.591 |
| W3 | 0.68 | 0.682 | 0.655 | | 0.672 |
| W4 | 0.658 | 0.666 | 0.708 | | 0.677 |
| W5 | 0.667 | 0.622 | 0.593 | | 0.627 |
| W6 | 0.689 | 0.666 | 0.607 | | 0.654 |
| W7 | 0.671 | 0.619 | 0.599 | | 0.630 |
| W8 | 0.693 | 0.672 | 0.623 | | 0.663 |
| W9 | 0.677 | 0.632 | 0.649 | | 0.653 |