CHAPTER THREE

3 PRESERVATION OF DISPERSED SYSTEMS

3.1 INTRODUCTION

Quality assurance is a major consideration in the manufacture of cosmetics, toiletries and all types of pharmaceuticals. One main concern with the aspect of product quality is the assurance there is no contamination with organisms, which might affect their safety, efficacy or acceptability to the consumer or patient. One obvious approach to this problem is to ensure all products are manufactured either as sterile products or as single-use packs. For cosmetics and toiletries and many pharmaceuticals, this is neither appropriate nor commercially viable and alternative means of microbial quality assurance are required. During product manufacture, microbial contamination is mainly controlled by the application of Good Manufacturing Practice (GMP), whilst the maintenance of quality during storage and use (also the responsibility of the manufacturer) are achieved largely by the inclusion of preservatives but also by product design and by product formulation and packaging. In practice, the presence of micro-organisms in pharmaceuticals, cosmetics and toiletries constitutes a potential hazard for two reasons. Firstly, it may result in spoilage of the product – the metabolic versatility of micro-organisms is such that almost any formulation ingredient may undergo degradation in the presence of a suitable micro-organism. Secondly, it may constitute an infection hazard to the consumer or patient, although here we have to bear in mind the degree of hazard will vary considerably from one situation to another, according to the intended use of the product (oral, topical, application to the eye, etc.) by the patient or consumer. In attempting to define acceptable standards or limits for microbial contamination for non-sterile products, it is therefore extremely difficult to establish what levels and types of contamination represent a hazard and what can be considered as safe (Bloomfield, 1996:3).

3.2 OBJECTIVES FOR PRESERVATION OF PHARMACEUTICAL PRODUCTS

Although the microbial quality of products should be achieved by GMP, it may be necessary in certain types of product to include a preservative to protect the product against residual contamination introduced during manufacture and any further microbial contamination which might occur during use. It should be clearly understood, however, that the function of the preservative must not be to cover bad manufacturing practice but to ensure that the product remains in a satisfactory condition during storage and use (Bloomfield, 1996:5).

In solving problems related to chemical preservation, there are four main questions which have to be answered (Bloomfield, 1996:5).

- Under what conditions is the inclusion of a preservative justified?
- What constitutes adequate preservation?
- Which preservatives are available to meet the requirements?
- How is it established that a product is preserved effectively?

In deciding the conditions for which the inclusion of a preservative is justified, in some situations the indications are obvious whilst in others it may be disagreeable. In principle, the preservation of dispersed pharmaceuticals is no different from the preservation of solution dosage forms. The complexity of such systems, however, necessitates special care in the selection and use of a preservative. Lotions and creams are especially favourable environments for microbes. Such formulations contain more than enough carbon, nitrogen and water for the metabolic needs of microbes.

In addition, the more complex a formulation (e.g. greater number of ingredients), the greater the likelihood that one or more ingredient(s) might inactivate the preservative. Dispersed formulations have to take in consideration the partitioning of preservatives between the two phases of an emulsion. Partitioning can reduce the concentration of a preservative in the aqueous phase, where it must be present for antimicrobial activity.

Another consideration is pH, which affects the efficacy of many preservatives. Since microbes live on a microscale, often living within water droplets or on the interfaces between phases, they experience only the local pH. The formulation scientist can either measure only the pH of the aqueous phase or an overall, ~~-a~~pparent" pH. This

latter measurement is subject to large errors resulting from the presence of organic phases and particles. An extra consideration is that particles are not always neutral and can donate or accept hydrogen ions. This results in local pH gradients that can affect microbial growth and preservative efficacy and stability. Another problem associated with preservation of dispersed systems is the effect of manufacturing on a preservative's efficacy in a formulation. The manufacturing processes for dispersed systems are especially complex and can cause the inactivation, volatilisation, degradation, or localisation of the preservative. A compound might meet the requirements of an ideal preservative for a specific formulation, yet will not be useful because of the problems associated with the required manufacturing processes (Anger *et al*., 1996:381).

It is necessary to consider what constitutes adequate preservation. As far as pharmaceuticals are concerned, it appears the licensing authorities currently allow themselves to be guided by criteria defined in the *British Pharmacopoeia (BP)*, *European Pharmacopoeia (EP)* and *United States Pharmacopoeia (USP)* for testing of preservative efficacy. The criteria appear to be based on knowledge of what is achievable in relation to the range of available preservatives and a working knowledge of preservative efficacy in manufactured products (Bloomfield, 1996:5).

In choosing a suitable preservative for a particular formulation, the compound is required to have certain characteristics (Bloomfield, 1996:6).

- It should be active against the required spectrum of organisms at a low concentration.
- It must be stable over a wide pH and temperature range.
- It must be soluble in the aqueous phase of the formulation.
- It must be stable and non-volatile.
- It must be compatible with formulation ingredients and packaging.
- It should be colourless, odourless and tasteless.
- It must be of low toxicity, non-sensitising and relatively inexpensive.

Although knowledge of the properties of a preservative may assist in the choice of a suitable system, it is recognised that selection based on theoretical considerations can be regarded only as a guide, since in many cases the interactions are incompletely understood. For this reason, it is always necessary to undertake microbiological tests to determine the efficacy of the preservative system for each individual product (Bloomfield, 1996:7).

3.3 SOURCES OF MICROBIAL CONTAMINATION

In order to control microbial contamination of pharmaceuticals, the sources and routes from which contamination may originate must be known. Raw materials may transfer microbial contamination to the product and further contamination may be introduced from the manufacturing equipment and environment, from the process operators and from the packaging materials (Bloomfield, 2007:26).

3.3.1 RAW MATERIAL

Both dry powders of natural and synthetic origin are mainly the bearers of aerobic spores with low bacterial count when used as raw materials in pharmaceuticals. They often contain gram-positive spore-formers and moulds but on occasions, they present with coliforms and gram-negative species. By far the most common source of spoilage or pathogenic organisms is water or unpreserved stock solutions, for example, solutions such as peppermint water may become heavily contaminated with gram-negative organisms if not properly prepared or if incorrectly stored (Bloomfield, 2007:26).

3.3.2 EQUIPMENT

Contamination may arise from a manufacturing and filling plant, which comes into direct contact with the product. In dead spaces (e.g. joints and valves) where water and product residues accumulate the growth of contaminants, most particularly gram-negative bacteria readily occur. Once established, this contamination can be very persistent and difficult to eliminate. Product containers and closures must be bacteriologically clean. With sterile fluids the containers should be adequately designed and constructed to protect the product (Bloomfield, 2007:27).

3.3.3 PERSONNEL

Contamination from process operators must be considered a significant hazard. During normal activity, loss of skin scales by shedding is about 10^4 min^{-1}. A large proportion of these skin scales will be contaminated with species of the normal skin flora, mainly non-pathogenic micrococci, diphterhoids and staphylococci, but may also include *Staphylococcus aureus*. Other organisms (e.g. *Salmonella and Escherichia coli*), not part of the resident skin flora, may also be carried transiently on the skin surface where poor hygienic practices exist among operators and may be shed into the product via skin scales or direct contact (Bloomfield, 2007:27).

3.3.4 CONSUMERS

Apart from contamination arising from raw materials and during manufacture, product storage and use may also contribute. A mixed microbial flora may be present in the product if the consumer who uses the product introduces bacterial contamination. Since the source of contamination can introduce pathogens, it is essential the preservative system is able to handle the bioburden the consumer can be expected to add (Anger *et al*., 1996:384).

Evaluating the results of surveys of ~~-in~~-use" microbial contamination, it must be borne in mind that contamination levels in used products reflect not only the bioburden introduced by the patient but also the survival characteristics of the contaminant in the product (Bloomfield, 2007:27).

3.4 FACTORS AFFECTING PRESERVATIVE ACTIVITY

3.4.1 WATER

Water is the single most important requirement for microbial growth. In a multiphase system, microbes will grow in only the aqueous phase (although they can collect at interfaces). Anhydrous pharmaceuticals will rarely support microbial survival unless extraneous water has entered the product container (Anger *et al*. 1996:384).

3.4.2 NUTRITION

Nearly all micro-organisms require certain basic nutrients to survive and grow in a pharmaceutical product. Many of these nutrients are found in dispersed dosage forms. These include carbohydrates, proteins, lipids (waxes, fatty acids or fatty alcohols), organic acids, inorganic salts and vitamins (Anger et al., 1996:385).

3.4.3 pH

The pH can have a limiting effect on the survival and growth of micro-organisms. Unfortunately, this occurs only at the pH extremes of below 3.5 or above 10. Few if any formulations are so acidic or basic. Bacteria are tolerant of pH and are able to thrive in a pH range of 5.5 to 8.5. Yeast and fungi prefer more acidic conditions of pH (e.g. 4 to 6). Microbial growth in formulations usually alters the pH and these changes often greatly affect preservative activity (Anger *et al*., 1996:386). Reduced pH drop observed in all emulsions containing parabens may support the hypothesis that there is an interaction of parabens with phospholipids and lipids in the interface region. As an example, the lipids may be protected from the attack of water molecules due to increased viscosity of the interface layer, as observed by Zhang

and Kirsch (2003:135). Alternatively, hydrolysis of parabens and the following dissociation of the product accumulated in the interface region may provide hydrogen ions which make the micro-environment more acidic, inhibiting hydrolysis of phospholipids and pH drop (Pongcharoenkiat *et al*., 2002:563).

3.4.4 TEMPERATURE

Storage temperature can greatly affect the survival and growth of microbes in pharmaceutical products. Although moulds and yeast prefer temperatures of 18 to 25°C, bacteria prefer 30 to 37°C; in both cases, many are inhibited and often destroyed by temperatures of 42 to 50°C. Storage of finished products at 23°C encourages moulds and yeasts to germinate and grow. These temperatures also tend to keep bacteria static, but alive and able to respond to future changes in temperature. Temperatures of 4 to 15°C tend to hold microbial contaminants in a state of dormancy however, the lower temperatures severely hamper the antimicrobial activity of preservatives. Conversely, even though higher temperatures increase the rate of microbial growth, they also increase the efficacy of a preservative, by 2 to 50 times for a 10°C increase in temperature (Anger *et al*., 1996:386).

3.4.5 INTERFACES AND ATTACHMENT SUBSTRATES

Suspended particles can adsorb microbes, this affords greater protection and, thereby, greater survival for them. Dispersed liquid antacid preparations are often found contaminated with gram-negative bacteria, including *Pseudomonas*. *Escherichia coli* adhesion to magnesium trisilicate was ascribed to van der Waals forces. In addition to the problem of protecting microbes, nutrients present in low concentrations in a formulation may be concentrated at particle surfaces and thus be available for attached microbes (Anger *et al*., 1996:386).

3.4.6 BIOBURDEN

The number of micro-organisms introduced into a formulation greatly affects the potential for product deterioration. Very low contamination levels, fewer than 10 microbes per gram, require a rather long lag period before growth or product degradation can be expected in an adequately preserved formulation. In contrast, very large inoculums may overwhelm a preservative system in a short time. Also, the greater the number of cells contaminating a formulation, the greater the chance that cells resistant to the preservative may develop. A weakness of most microbiological testing programmes is the failure to detect low levels of contaminants, which after a

three-month storage period may grow into thousands of organisms per millilitre (Anger *et al*., 1996:387).

3.4.7 PRODUCT INGREDIENTS AS PRESERVATIVES

In a few cases, a product is self-preserving without an additional preservative. Alcohol is bactericidal at concentrations over 20% and used in many dispersed systems, although high concentrations strongly affect the physical properties of the product. Another example is glyceryl monolaurate, a useful non-ionic surfactant that also has bactericidal activity (Anger et al., 1996:387).

3.4.8 POTENTIATION AND SYNERGY

Significant practical benefits and advantages can be gained by the use of preservative combinations; these include:

- An increased spectrum of activity.
- The use of lower concentrations of individual components resulting in a possible reduction in toxicity.
- The prevention of the development of microbial resistance to individual preservatives.
- A possible overall enhancement of antimicrobial activity, beyond that expected from simple addition (synergy).
- An extended time course of preservation achieved by combining a labile, markedly biocidal preservative with a stable longer-acting agent.

Careful selection of individual agents for combination, based on their physicochemical properties, may serve to overcome microbiological problems created by the physical limitations of individual preservative agents (Denyer, 1996:134).

Darwish and Bloomfield (1996:51) added three co-solvents to formulations with methyl and propyl para-hydroxybenzoate and tested the combined effect on preservative efficacy. Results obtained indicated the inclusion of a co-solvent has the potential to increase the usefulness of the parabens as preservative agents for pharmaceuticals, cosmetics etc., by facilitating an increase in aqueous concentration above their saturation solubility.

3.5 CONSEQUENCES OF MICROBIAL CONTAMINATION

3.5.1 AESTHETIC MANIFESTATION

If ambient conditions encourage microbial growth, changes in the product may be an inevitable consequence of the metabolism of the multiplying organisms. These changes may be detectable by one or more of the senses.

3.5.1.1 Visible effects

Homogeneous or colonial growth in or on a product probably constitutes the most striking and frequent manifestation. Contaminants may be seen as sediment, turbidity or pellicle in liquid products and in more solid preparations, coloured colonies may form. Growth is not as readily detectable in suspensions, except at the surface, because of inherent opacity, but preparations of this type can thin, separate, decolour or change color because of microbial contamination. The same sort of change can occur in emulsions which may become visibly heterogeneous owing to hydrolysis of the oil phase or changes in pH of the aqueous. Mould growth is one of the most common visible manifestations of spoilage in creams and can often be attributable to the containers used for storage. Spores in dust particles, if not properly removed, may germinate and grow on the inside of the filled containers. Condensation in large air spaces of jars, owing to fluctuating storage temperatures, may also provide suitable conditions for the growth of spores initially present, perhaps on the inner surface of the closure (Spooner, 1996:18).

3.5.1.2 Olfactory effects

A variety of aroma-producing bacteria have long been identified. Often their unpleasant odours are combined in spoiled products and are particularly disastrous in cosmetics and toiletries, which depend so much on their own specific perfumes (Spooner, 1996:19).

3.5.1.3 Taste

Reports that oral products taste ~~p~~eculiar" are sometimes the first indication that spoilage has occurred. However, a patient may not consider an unpleasant flavour significant, as medicines are traditionally believed to be disagreeable (Spooner, 1996:19).

3.5.1.4 Tactile effects

The texture of topical products is vital to acceptability. Contaminated creams may become lumpy and changes in the viscosity of contaminated liquids can be detected when applied to the skin (Spooner, 1996:19).

3.5.1.5 Audible effects

The growth of organisms, even in heavily contaminated products, cannot be heard, but their gas production may give rise to readily detectable sounds (Spooner, 1996:20).

3.5.2 TOXICITY

There is a rather more sinister aspect of spoilage if it is not detected organoleptically. The production of toxins or metabolites, or the inactivation of biologically active constituents in a formulation can cause harm to consumers. Toxic metabolites may be released by some species of microorganisms, which may render the product dangerous to the patient. An example in relation to pharmaceutical products is pyrogens. These are mainly lipopolysaccharide components of gram-negative bacterial cell walls, which can cause acute febrile reactions if introduced directly into the bloodstream. These toxins are heat stable and may be present even when viable organisms are no longer detectable. They are, however, only poorly adsorbed via the gastrointestinal tract and are therefore of little importance in oral preparations (Bloomfield, 2007:32).

3.5.3 DEGRADATION OF ACTIVE CONSTITUENTS

Spoilage, which may involve phenomena such as creaming of emulsions, cracking, viscosity changes or separation of suspended material, is seen as the result of a general breakdown of the formulation. Complex formulations are particularly prone to this type of spoilage. Many of the surfactants used in pharmaceutical emulsions are subject to microbial degradation, particularly the non-ionic surfactants. Ability to degrade surfactant molecules is again limited to a small range of organisms, particularly the *Pseudomonas* species (Bloomfield, 2007:33).

Osmophillic yeasts are known for microbial attack on sugars and other sweetening agents. Oral suspensions or emulsions containing sugars are liable to ferment with production of gas and acid, which may be sufficient to alter the stability of the formulation (Bloomfield, 2007:35).

3.6 BACTERIAL MORPHOLOGY

Bacteria are a major group of unicellular living organisms found in a wide variety of shapes belonging to the prokaryotes. Eubacteria's cell structures are relatively simple and lack cell nuclei but possess cell walls, which provide structural integrity to the cell. The structure of bacteria differs from all other organisms due to the presence of peptidoglycan, which provides rigidity of the bacterial cell wall and determines the cell shape. The peptidoglycan is located directly outside the cytoplasmic membrane. The cell walls of all bacteria are not identical, in fact the cell wall composition is one of the most important factors in the analysis and differentiation of bacterial species. Accordingly, two general types of bacteria exist, of which Gram-positive bacteria are comprised of a thick peptidoglycan layer connected by amino acid bridges (Figure 3.1). On the contrary, the cell wall of Gram-negative bacteria is much thinner and composed of only 10 to 20% peptidoglycan. In addition, the cell wall contains an additional outer membrane composed of phospholipids and lipopolysaccharides (Vijayaraghavan & Yun, 2008: 271).

Gram-positive and Gram-negative organisms possess structural dissimilarities but also striking differences between the cell wall compositions. The walls of Gram-positive organisms, after removal of surface protein, contain three or four amino acids, whereas the walls of Gram-negative organisms contain a variety of amino acids comparable to those found in proteins. Many Gram-positive organisms possess higher amino sugar content and, in general, a much lower lipid content than Gram-negative organisms. Nucleic acids are not present in the isolate cell wall of either type of organism (Kabara, 1984:25).

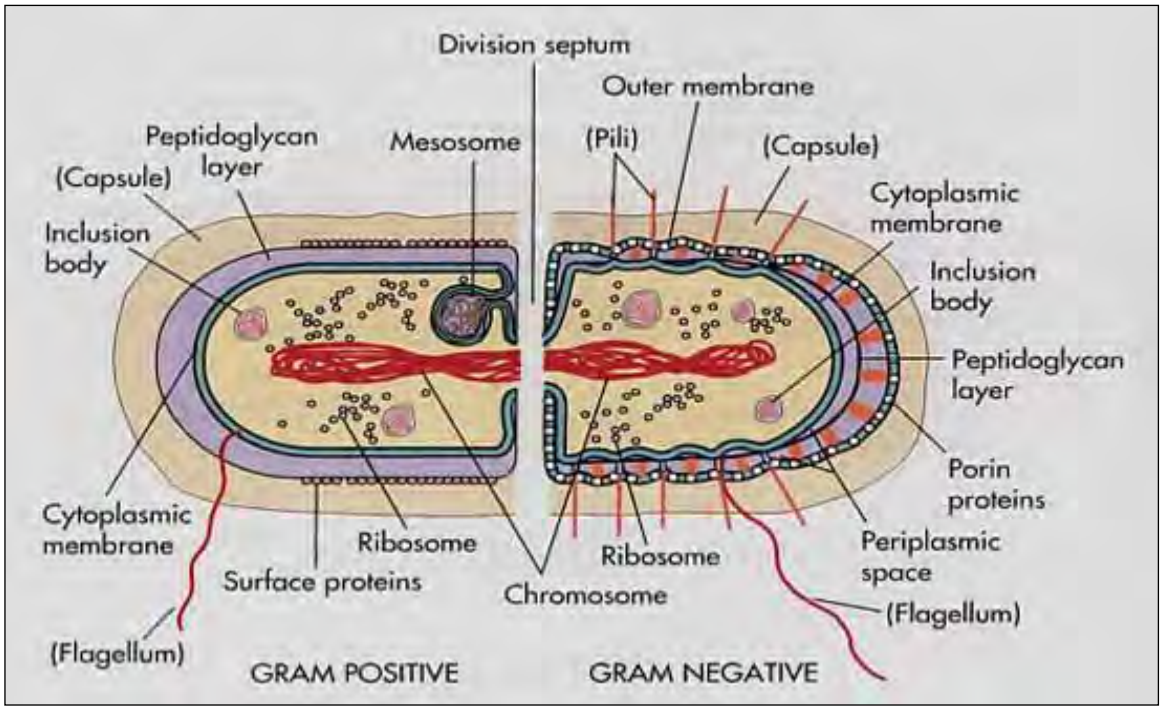

Figure 3.1: Structure of Gram-positive and Gram-negative bacteria (MEDICAL MICROBIOLOGY, 2002).

3.6.1 POSSIBLE INTERACTION OF PHEROID® COMPONENTS ON BACTERIAL CELL STRUCTURE

The Pheroid® is capable of penetrating skin, keratinised tissue, intestinal epithelium, vascular walls, sub cellular organelles, sensitive and resistant parasites, bacteria and fungi. Research has not only shown effective penetration of these last organisms but also the capability of the Pheroid® to deliver drugs to these organisms and destroy them (Grobler, 2004:13).

The main components of the Pheroid® are the water phase and the oil phase, which constitutes unsaturated fatty acids, the pegylated ricinoleic acid, nitrous oxide and α-tocopherol. Figure 3.2 gives a schematic model of the components in the Pheroid® formula.

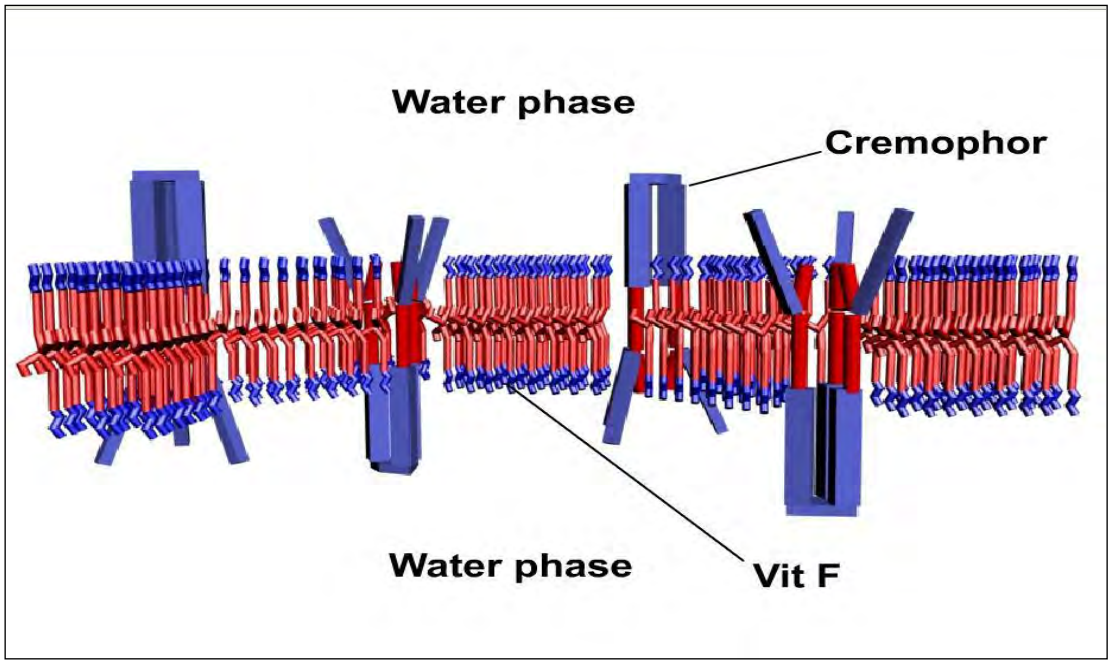

Figure 3.2: A schematic model of the fatty acid components of the membrane of the Pheroid®. The blue regions represent the hydrophilic domains whereas the red regions represent the hydrophobic domains. Each fatty acid contained in vitamin F (Vit F) is thus sketched as a red hydrocarbon chain with a blue ethyl ester attached. The hydrocarbon chains are bent where unsaturated C=C bonds occur. The pore structures or channels are formed by the Cremophor molecules. The nitrous oxide and α-tocopherol are not, as yet, accommodated in the model (Grobler, 2009:195).

In order to evaluate the ability of Pheroid® to sustain growth inhibition of *M. tuberculosis* H37Rv, duplicate cultures (Figure 3.3) were treated with a single administration of Pheroid® (1:40 final dilution) and incubated at 37˚C for seven days, at which stage the cultures became BACTEC positive. An untreated *M.tb.* culture was included to follow normal growth and acted as control. A repeat Pheroid® administration was added to one of the initial Pheroid®-treated duplicate cultures on day seven and the incubation was continued. Figure 3.3 shows that the initial treatment of the culture (curve B) resulted in a 93% inhibition in mycobacterial growth during the first seven days of incubation compared to the control untreated culture (curve A), after which the culture grew exponentially up to day 12. However the duplicate culture treated with a second Pheroid® administration showed additional inhibition of growth (Fig. 3.3 curve C) with inhibition of growth up to day 12 of incubation (GI=163) (Grobler, 2009:213).

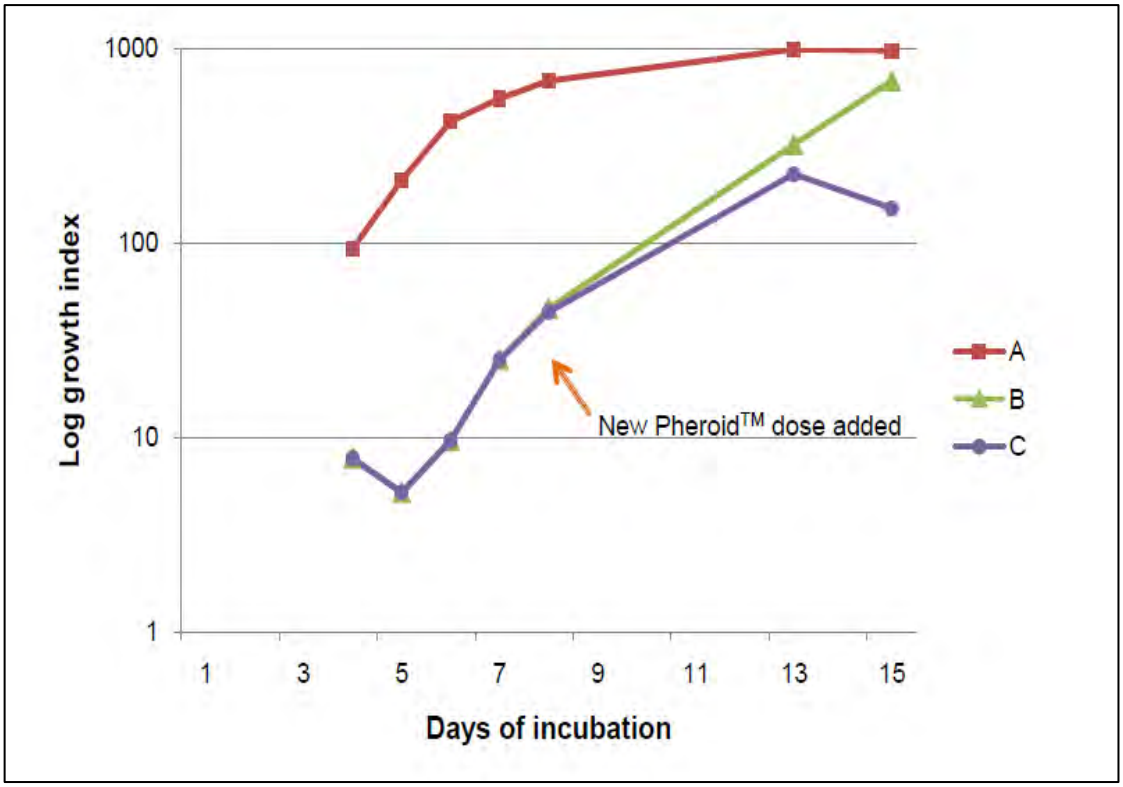

Figure 3.3: Impact of Pheroid® on M.tb. growth.

M.tuberculosis H37 RV was treated with Pheroid® (1/40 dilution) in two parallel cultures (B and C). Both cultures were simultaneously inoculated from a single primary culture. One of the cultures (C) was treated with a supplementary dose of Pheroid® on day seven of incubation and both cultures (B and C) were incubated to day 12. An untreated culture (A), inoculated from the same stock as B and C, was included to monitor culture growth (Grobler, 2009:214).

Subcomponents of Pheroid® were evaluated to establish the origin of the inhibitory effect observed. The nitrous oxide-saturated essential fatty acid fraction (1.95% of the total Pheroid® preparation) showed the same inhibitory effect described above, as did the complete formulation, whereas the nitrous oxide gassed water fraction (82.8% of the whole suspension) showed no effect on growth compared to the control. These results may be interpreted to mean:

- Nitrous oxide plays no role in the mycobacterial growth inhibition.
- The nitrous oxide in the water is diluted upon addition to the bacterial growth medium, since a number of salts cause dissipation of the gas from the water phase.
- The fatty acid fraction itself interferes with the bacterial growth.

- Sufficient nitrous oxide is entrapped within the fatty acid phase and nitric oxide groups are available to result in growth inhibition.
- Probably a combination of more than one of these possibilities.

In two separate studies performed, the bactericidal effect of supercritical N_2O on *Staphylococcus aureus, Eschericia coli and Pseudomonas aeruginosa* were examined. Mun *et al*. (2012:15) found approximately a 7-log of both *Staphylococcus aureus* and *Eschericia coli* by supercritical N_2O were completely inactivated within 20 minutes, although *Staphylococcus aureus* exhibited relatively low sensitivity to supercritical N_2O compared to *Eschericia coli*. The bactericidal efficiency was not adversely affected by varying the suspending medium and the presence of oleic acid enhanced the inactivation. Mun *et al*. (2011:372) achieved an 8-log reduction of *Pseudomonas aeruginosa* cell concentration in neutral phosphate-bufferd saline by treatment with the supercritical N_2O. No pH changes were found in the presence of vigorous mixing (600 rpm) within six minutes, in a condition of 37°C and 10 MPa.

3.6.2 MODE OF ACTION OF PARABEN PRESERVATIVES ON BACTERIA

Various researchers have suggested the mechanism of action of parabens relies mainly on the disorganisation of the microbial cell membrane. At low (bacteriostatic) concentrations, parabens appear to inhibit the uptake of metabolites, whilst at higher (bactericidal) concentrations, loss of the membrane semipermeability occurs. Hansch *et al*. (1972:428) concluded that the activity of the membrane active antibacterials such as the parabens depends on their ability to move freely in the aqueous phase and yet be lipophilic enough to partition through the microbial outer cell envelope (where present) and the cytoplasmic membrane. Studies done by Hugo and Russell (1982:23) and Russell and Chopra (1996:101), show that various alcohol and glycol compounds also have antimicrobial activity which is associated with damage to the cytoplasmic membrane. From the various studies described above, a number of possible explanations for the observed effects of the co-solvents on the activity of the parabens can be proposed:

- The co-solvent may interact with the parabens in the aqueous phase thereby altering the hydrophilic/lipophilic balance and affecting their uptake and/or interaction with the cell.
- Since the co-solvents on their own cause damage to the cell membranes, potentiation of antimicrobial action may result from a direct effect on the microbial outer cell envelope (where present) or cytoplasmic membrane.

- The generally higher resistance of gram-negative bacteria to parabens as reported by a number of researchers (Hugo and Russell, 1982:23: Russell and Chopra, 1996:101) is usually attributed to the presence of the outer cell membrane which hinders access of the parabens to the cytoplasmic membrane. Uptake of parabens through the gram-negative outer cell membrane is thought to occur by the hydrophobic route.
- The outer membrane (OM) of Gram-negative bacteria provides an effective barrier to their often-harsh extracellular milieu. In particular, the outer leaflet of the OM is not a canonical monolayer of phospholipids, but is composed of lipopolysaccharide (LPS), a molecule generally consisting of a core of Lipid A decorated with inner and outer core oligosaccharides. The oligosaccharides extend ~30 Å above the plane of the lipid headgroups of the outer leaflet. As such, it is an effective permeability barrier against potentially harmful compounds. However, permeability is required for bacterial survival; no bacterium is an island, as it were. For example, uptake of nutrients is essential and OM transport proteins are required to conduct this function. Virtually all OM proteins are β-barrels, consisting of an even number of eight to twenty-four of β-strands forming a pore-like structure. Many of these OM pore-like β-barrels are classified as porins and most nutrient uptake is accomplished by them. The effective aperture of the porin is dictated by the number of β-strands and the aperture size then dictates the size (and shape) of the solutes that can diffuse through them. Porins function passively, permitting the energy-independent diffusion of solute molecules with a molecular mass of 600 Da or less downhill across a concentration gradient, through the porin's β-barrel, and into the periplasm. Another class of energy-independent OM transporters uses low-affinity binding sites that effectively serve to amplify small concentration gradients at the site of the transporter (Wiener and Horanyi, 2011).
- Since the co-solvents are known to disrupt the cytoplasmic membrane it might be expected they could also interact with the gram-negative outer cell membrane, thus facilitating access of parabens to the cytoplasmic membrane. They are relatively small hydrophilic molecules and it is likely that these molecules pass freely across the outer membrane though the porins (i.e. the hydrophilic routes) to reach the cytoplasmic membrane where they exert their action (Russell and Chopra, 1996:101).

3.7 FUNGI MORPHOLOGY

Fungi are eukaryotic organisms, i.e. their cells possess a nuclear membrane. Many similarities are found between the biochemistry of fungal cells and human cells. The typical yeast cell has an oval shape and is surrounded by a rigid cell wall. The cell wall contains a number of structural polysaccharides and may account for up to 25% of the dry weight of the cell wall. Removal of the cell wall leaves an osmotically fragile protoplast. Most of the cell's genome is concentrated in the nucleus, which is surrounded by a nuclear membrane that contains pores to allow communication with the rest of the cell (Figure 3.4). Actively respiring fungal cells possess a distinct mitochondrion, which has been described as the power-house of the cell. In addition, the vacuole acts as a "storage place" where nutrients, hydrolytic enzymes or metabolic intermediates are retained until required (Kavanagh and Sullivan, 2004:44).

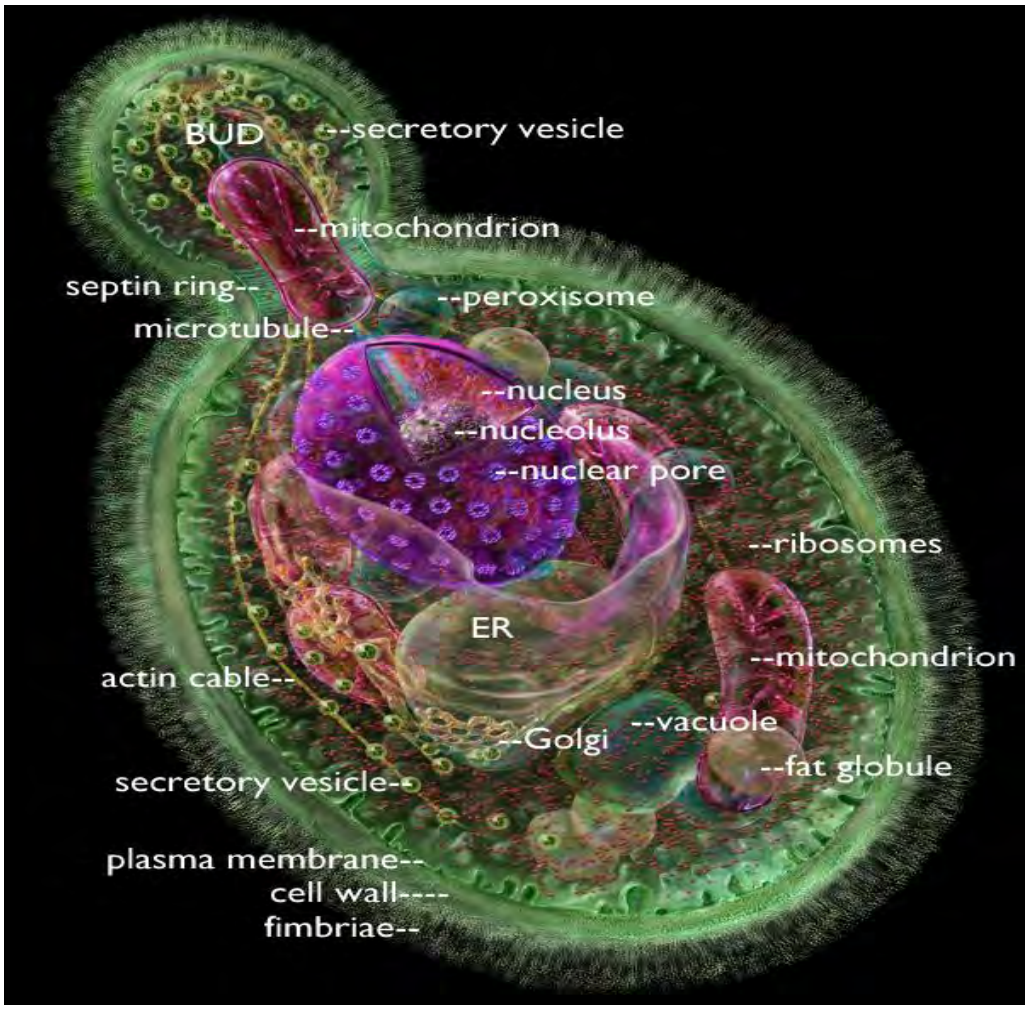

Figure 3.4: Internal structure of a budding yeast cell (Copyright Russel Kightley, 2013).

3.8 EVALUATION OF THE EFFECTIVENESS OF ANTIMICROBIAL PRESERVATION OF EMULGEL FORMULATIONS

To determine the efficacy of antimicrobial preservatives in medicinal products the inhibition of growth of microorganisms must be measured in conjunction with several factors that could influence the stability of the preservative. Although the chemical, physical and microbiological properties of a preservative are clearly important, they do not provide sufficient information to predict a formulated product will be adequately preserved. A challenge test (also known as a preservative effectiveness test (PET) or antimicrobial effectiveness test) is a procedure to determine whether a formulated cosmetic, pharmaceutical or other type of product is adequately preserved to prevent proliferation from raw products and during consumer use. Different Pharmacopoeias, for instance the EP, BP and USP contain methods intended for the assessment of the efficacy of an antimicrobial preservative in pharmaceutical products. These methods vary only slightly from one Pharmacopoeia to another and basically share the following experimental procedures. The product for testing was inoculated with five different strains of microorganisms (bacteria and fungi). The contaminated product was sampled at different, well-defined time intervals and the number of viable organisms determined. The preservative must cause a specified reduction in viable count, which must be maintained over a period of 28 days (Kramer *et al*., 2008:547).

3.8.1 CHOICE OF TEST ORGANISMS AND POSITIVE IDENTIFICATION

In accordance with the Pharmacopoeias, the following strains of microorganisms were used: *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739) and *Aspergillus niger* (ATCC 16404).

Strain ATCC 16404, currently known as *Aspergillus niger*, was designated as a quality-control reference strain in a number of applications. It is also cited as the standard culture in several official methods (USP) and manuals, as well as the Code of Federal Regulations. Recently, a polyphasic study was performed at American Type Culture Collection (ATCC) in which molecular data was combined with physiological characteristics. The results clearly indicated that ATCC 16404 is a member of the unique species *Aspergillus brasiliensis*, thus it was renamed ATCC 16404 *Aspergillus brasiliensis* (Lyons, 2013).

3.8.1.1 Gram-negative organisms

Gram-negative organisms include *Pseudomonas aeruginosa* and *Escherichia coli*.

Pseudomonas aeruginosa (Figure 3.5) has acquired some notoriety as a contaminant of pharmaceutical products, especially eye preparations. It is a flagellated, rod-shaped organism that does not form spores and grows aerobically. It does not ferment carbohydrates and thus will not produce gaseous products when growing in the presence of carbohydrates. Under appropriate conditions of growth, it may produce a blue or green fluorescent media. As a mesophile, *Pseudomonas aeruginosa* will grow in a temperature range of 20 to 42°C with optimal growth occurring at 37°C (Baird and Denyer, 2007:18).

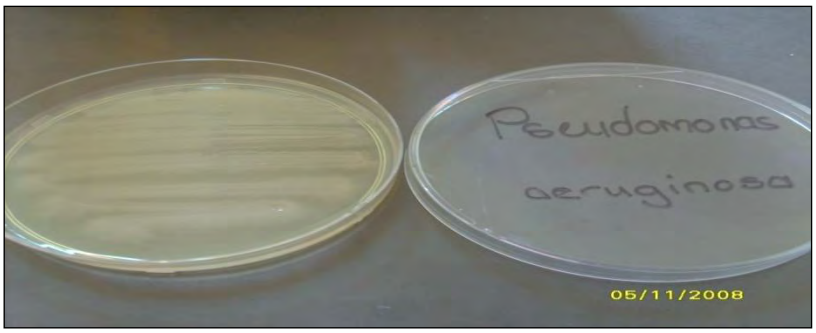

Figure 3.5: Petri dish with *Pseudomonas aeruginosa* colonies.

Escherichia coli (Figure 3.6), a member of Enterobacteriaccea, are a motile, non-sporin rod with typical of dimensions 1 µm x 4 µm. It differs from *Pseudomonas aeruginosa* in that it is able to grow anaerobically. When it grows on carbohydrates it does so by fermentation, producing gaseous products. It can grow at low temperatures, but also at temperatures as high as 40°C. Optimal growth occurs at 37°C (Baird and Denyer, 2007:18).

Figure 3.6: Petri dish with *Eschericia coli* colonies.

3.8.1.2 Gram-positive organisms

Staphylococcus aureus (Figure 3.7) represents a gram-postitive organism. This is a spherical organism of approximately 1 µm in diameter, non-motile and does not form spores. It is able to grow aerobically and anaerobically and will grow readily in a chemically defined medium containing glucose, essential salts, selected amino acids, thiamine and nicotinic acid. It is relatively resistant to antimicrobial preservatives such as phenol and can remain alive at temperatures as cold as 4°C and as warm as 60°C (Baird and Denyer, 2007:19).

Figure 3.7: Petri dish with *Staphylococcus aureus* colonies.

3.8.1.3 Fungi

Two organisms fall in the class of fungi namely *Aspergillus brasiliensis* and *Candida albicans*.

Aspergillus brasiliensis (Figure 3.8) grows only in the filamentous (mycelial) form and is familiar to most people as a white, turning to black, disks of growth on jams and other exposed foodstuffs. Colonies grow over a wide temperature range, up to 50°C, although optimal temperature for growth is 24°C. Spores of Aspergillus are commonly present in air and can infest and germinate in pharmaceutical and cosmetic products, causing discolouration and spoilage. They are generally not as resistant to antimicrobial agents as are bacterial spores. Some Aspergillus strains produce characteristic carcinogens commonly known as aflatoxins (Baird and Denyer, 2007:20).

Figure 3.8: Petri dish with *Aspergillus brasiliensis* colonies.

Candida albicans (Figure 3.9), is a yeast which may cause oral and vaginal thrush. It grows readily on conventional mycological media at room temperature (optimal growth at 25°C) or at 37°C. It is dimorphic, first growing as yeast cells, but with aging will form chlamydospores, which are more difficult to destroy. There are no temperature tolerance differences between the two forms. Viewed microscopically, it appears to possess septate hypae, known as pseudomycelia, among the yeastlike cells. It is unpigmented and colonies have a creamy white appearance (Baird and Denyer, 2007:20).

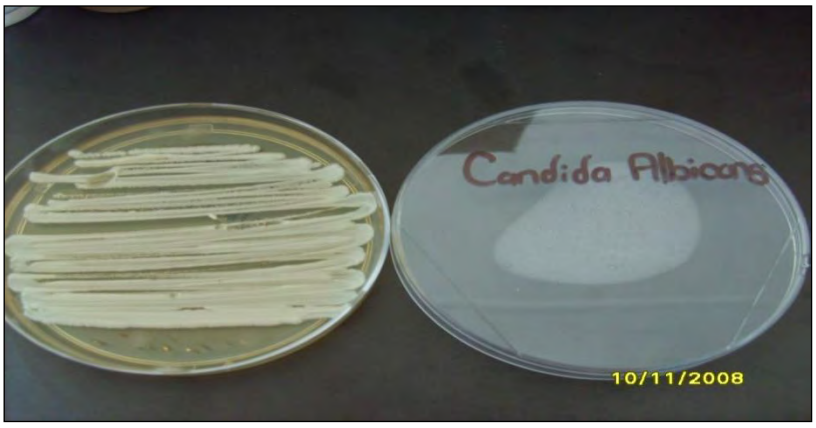

Figure 3.9: Petri dish with *Candida albicans* colonies.

3.8.2 MEDIA

Tryptone Soya Agar (TSA) plates served as growth media for the bacterial test cultures. The preparation was performed aseptically to prevent contamination of the plates. The method involved dissolving 38 g of TSA powder into one litre of distilled water and shaking well until the powder was fully dissolved. The mixture was placed in the Hirayama autoclave, under the liquid sterilisation cycle, for 15 minutes at 21°C. The warm mixture was removed from the autoclave and carefully poured into 90 mm

plastic petric plates. The plates were left overnight to cool down and then stored in the fridge until used for the preservative efficacy test. Any plates with growth on them after this storage period were discarded.

Media for yeast and moulds often have a lower pH (5.5 - 6.0) than bacterial culture media (7.0 - 7.4) hence Sabaroyed Dextrose Agar (SDA) plates served as growth media for *Candida albicans* and *Aspergillus brasiliensis*.

3.8.3 PREPARATION OF STANDARD GROWTH CURVES

The surface of three TSA plates, were incubated for the bacteria and two SDA for the yeast and fungi. The bacterial cultures were incubated at 30 to 35°C for 18 to 24 hours and *Candida albicans* at 20 to 25°C for 48 hours. *Aspergillus brasiliensis* was incubated at 20 to 25°C for seven days or until good sporulation was obtained. Sterile fluid containing 0.9% w/v sodium chloride and 0.1% w/v peptone was used to harvest the bacterial and Candida cultures.

Aspergillus brasiliensis was harvested with 0.9% w/v sodium chloride and 0.05% v/v Tween 80. The dilutions of the bacterial cultures and *Candida albicans* were made with sterile 0.9% w/v sodium chloride and 0.1% w/v peptone (also used as blank in the spectrophotometer). The dilutions of the *Aspergillus brasiliensis* culture were made with sterile 0.9% w/v sodium chloride and 0.05% v/v Tween 80 (also used as blank for this organism in the spectrophotometer).

The absorbencies of these dilutions were read with a Spectroquant® Merck™ - Pharo 300 spectrophotometer at 600 nm. At least five readings were taken for each organism and at each reading, dilutions were made of the culture and a triplicate standard plate count was performed in the order 10^{-1} to 10^{-8}. The aim of this procedure was to determine the absorbencies for each of the specific organisms, which represent approximately 10^{8} c.f.u. (colony forming units)/ml.

See Addendum D for standard growth curves of all five test organisms.

3.8.4 PREPARATION OF INOCULUM

The surface of three TSA plates, were incubated for the different bacteria respectively and two SDA plates for the yeast and fungi respectively. The bacterial cultures were incubated at 30 to 35°C for 18 to 24 hours and *Candida albicans* at 20 to 25°C for 48 hours. *Aspergillus brasiliensis was incubated* at 20 to 25°C for seven days or until good sporalation was obtained. Sterile suspending fluid containing 0.9% w/v sodium chloride and 0.1% w/v peptone was used to harvest the bacterial and *Candida albicans* cultures. *Aspergillus brasiliensis* was harvested with 0.9% w/v

sodium chloride and 0.05% v/v Tween 80. A volume of 2ml of this fluid was transferred onto the agar plate and where necessary the growth from the plate were scraped with a sterile inoculating loop. Thereafter the growth was transferred into a suitable vessel. The c.f.u. count was adjusted to approximately 10^8 per ml by using the standard curve prepared and using the same sterile suspending fluid used for harvesting. The suspensions were used immediately.

3.8.5 TEST PROCEDURE

The test consisted of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples.

The preservative properties of the preparation were adequate if, in the conditions of the test, there was a significant decrease or no increase, as appropriate, in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The criteria of acceptance, in terms of decrease in the number of micro-organisms with time, vary for different types of preparations according to the degree of protection intended. Throughout the test procedure for each individual preservative under investigation the following criteria were applied:

- Control for each organism was 100 ml of either Peptone or Tween water inoculated with (200 µl) of each stock suspension of organisms.
- The test samples were inoculated with (200 µl) of the stock suspensions per 100 ml of Pheroid® formulation prepared with the appropriate preservative under investigation.
- At time 0 hours the samples were diluted in the order of 10^{-3}, 10^{-4} and 10^{-5} for the bacteria and in the order 10^{-2}, 10^{-3} and 10^{-4} for *Candida* and *Aspergillus*.
- At time 14 and 28 days the samples were diluted in the order of 10^{-1}, 10^{-2} and 10^{-3} for all five organisms.
- A volume of (100 µl) of the diluted samples was removed and plated in triplicate on the agar plates.
- The criteria for log reduction for oral preparations were followed (BRITISH PHARMACOPOEIA, 2013).

3.8.6 PLATE-COUNT METHOD

Figure 3.10 portrays the procedure followed. A known volume, usually 200 µl of a suitable diluted culture was pipetted on to an over-dried agar plate and distributed

evenly over the surface using a sterile spreader made of wire or glass capillary. The liquid was allowed to soak in before the plates were inverted. A series of tenfold dilutions was made in a suitable sterile diluents and replicates plated out at each dilution, in order to ensure that countable numbers of colonies (30 to 300) were obtained per plate. The viable count was calculated from the average colony count per plate, knowing the dilution and the volume pipetted onto the agar.

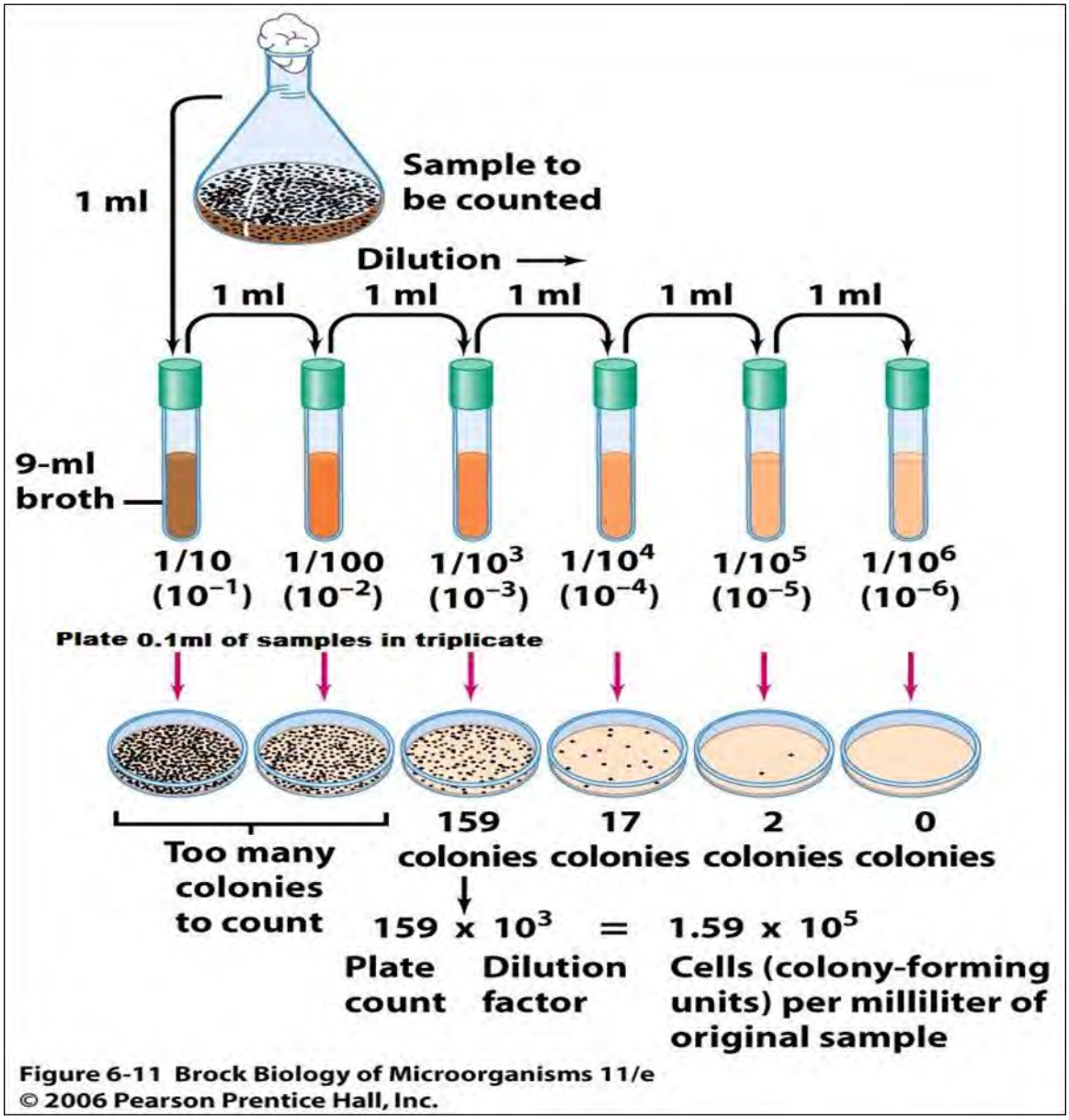

Figure 3.10: Serial dilution scheme (Ekanayake, 2012).

Example calculation:

Stock bacterial suspension, 1 ml added to 9 ml of sterile diluent – labeled dilution A (the stock solution has therefore been diluted by a factor of 10 (10^{-1}).

1 ml of dilution A added to 9 ml of sterile diluents – labelled dilution B (dilution B has been diluted by a factor of 10^{-2}).

1 ml of dilution B added to 9 ml of sterile diluent - labelled dilution C (dilution C has been diluted by a factor 10^{-3}).

1 ml of dilution C added tot 9 ml of sterile diluent – labeled dilution D (dilution D has been diluted by a factor of 10^{-4}).

1 ml of dilution D added to 9 ml of sterile diluent – labeled dilution E (dilution E has been diluted by a factor of 10^{-5}).

100 µl of each dilution were plated in triplicate.

Mean colony counts for each dilution after incubation at 37°C:

Dilution A	too many to count
Dilution B	too many to count
Dilution C	159 colonies
Dilution D	17 colonies
Dilution E	2 colonies

The result for dilution B was unreliable, as the count was too high. If the colony count exceeded 300, errors arose because the colonies were very small and could be missed. Similarly, the count for dilution D was unreliable because at counts below 30, small variations introduce high percentage errors.

The result from dilution C was therefore taken for calculation, as the colony count was between 30 and 300.

159 colonies in 100 µl, therefore this was diluted by a factor of 10^3 and so the count in the stock suspensions was $159 \times 10^3 = 1.59 \times 10^5$ cfu/ml.

3.8.7 CRITERIA OF ACCEPTANCE

To assay the surviving microorganisms of the microbial challenge, an aliquot of the contaminated product was withdrawn and diluted and microbial organisms were allowed to grow on or in appropriate growth medium at an appropriate temperature. A proper control for these experiments was an identical challenge test on the same

formulation without preservatives. At assay times, 1g or 1ml of product was aseptically removed, added to 9 ml of dilution broth and mixed well. More sequential 1:10 dilutions of this suspension were made in dilution broth. The number of microbes per millilitre of each of the dilutions was determined (in triplicate) by spreading 0.1 ml of the suspension on an agar plate. The plates were incubated at 35 °C for 48 to 72 hours for bacteria and *Candida* species and seven days for *Aspergillus*. The number of colonies on the plate was counted and the presence or absence of growth was recorded.

The criteria for evaluation of anti-microbial activity are given in terms of the log reduction in number of viable microorganisms using as baseline the value obtained for the inoculums (see Table 3.1). The *Pharmacopoeias* give the criteria for preservation of oral products. According to these criteria, oral preparations can be classified as safe if a log 3 reduction in counts (cfu) can be seen for bacteria within 14 days. Furthermore, no further significant increase in cfu's must be observed. For fungi the log reduction should be log 1 with no further increase in cfu.

Table 3.1: Oral preparations *(NI = No Increase)

	Log reduction	
	14 days	**28 days**
Bacteria	3	NI*
Fungi	1	NI*

3.8.8 PRESERVATIVE EFFICACY TEST PROCEDURE FOR EMULGEL FORMULATIONS

3.8.8.1 Materials

See Table 3.4 for the outlay of the preservatives used to preserve individual formulations of the Pheroid®. The synergistic effect of PG added as co-solvent to the Pheriod® formulations will be portrayed in the results obtained with the preservative efficacy tests performed on each formulation over a test period of 28 days. Nipasept® and Nipastat® were kindly donated by Clariant™. These formulas give the advantage of overcoming possible resistance of the organisms against single preservative components. Table 3.2 gives the individual components of these two combination products available on the market.

Table 3.2: Experimental outlay of the preservatives tested during the preservative efficacy test.

Preservative	*Concentration*	*Actives*
Control with no preservative added	0%	None
PG	10%	PG
Methylparaben	0.10%	Methylparaben (MP)
Methylparaben + PG	0.10% + 10%	Methylparaben and PG
Ethylparaben	0.15%	Ethylparaben (EP)
Ethylparaben +PG	0.15% + 10%	Ethylparaben and PG
Propylparaben	0.015%	Propylparaben (PP)
Propylparaben + PG	0.015% + 10%	Propylparaben and PG
Butylparaben	0.03%	Butylparaben (BP)
Butylparaben + PG	0.03% +10%	Butylparaben and PG
Nipasept®	0.175%	MP, EP and PP
Nipasept® + PG	0.175% + 10%	MP, EP and PP
Nipastat®	0.175%	MP, EP and PP, BP and Isobutylparaben
Nipastat® + PG	0.175% + 10%	MP, EP and PP, BP and Isobutylparaben

3.8.8.2 Methods of formulation

The preparation methods used to prepare each emulgel formula for evaluation of the preservative efficacy test can be found in Annexure E.

The basic Pheroid® formula was used, as described in Chapter 2 and the chosen concentration of preservative to be used in each formula were added in the water phase of the formula. As noted before, there is a decrease in solubility in water with an increase in alkyl group length from methylparaben through to butylparaben. Concentrations of the preservative used in the preservative efficacy tests were obtained from the Handbook of Excipients, Rowe *et.al*. (2003: 65, 244, 386, 526). The technical information for the combination preparations used was supplied by Clariant™.

Special precaution was taken not to overheat the mixture of water and paraben above 80°C, as chemical breakdown takes place at temperatures higher than this. Another method of incorporation involves the preparation of stock solutions of the paraben esters in solvents such as PG. In this method, a specified volume of the stock solution must be added slowly to the aqueous phase of the formulation in order to prevent precipitation of the ester (Haag & Loncrini, 1984:71).

The basic Pheroid® formula, without any preservative added, was prepared and tested as a control. The usefulness of PG as a possible preservative on its own and in combination with other parabens was investigated.

3.8.8.3 Results

Particle size

See Annexure F for Table F1 and Table F3 containing the particle size measurements for all the Pheroid® formulations in the preservative efficacy test. At stability interval 48 hours, the formulations without added organisms were measured and the D (0.9) values captured. D (0.9) represents the value below which 90% of the particles size range falls. After 28 days, the measurements for each formula with the five test organisms under investigation were taken and compared to the values initially obtained.

First, all the formulations exhibited the same overall size distribution, i.e. a relatively narrow non-Gaussian distribution. The average d (0.90) values for all the formulations manufactured, ranged below 0.3 µm. In general, the addition of the five test organisms did not influence the particle size measurements obtained at 28 days. The only formulation which exhibited an increase in the particle size measured was

the Pheroid® with 10% (v/v) PG added. The initial D (0.9) value measured at 48 hours ranged from 0.795 to 1.025 µm, the added bacterial strains altered the D (0.9) values between 2.216 to 3.691 µm and the added fungi and yeast between 0.358 to 1.025 µm. A possible explanation for the increase in the particle diameters could be that the added PG influenced the emulsification process during manufacturing. The influence on stability of this formula will be monitored for the remainder of the study.

pH

Annexure F for Table F2 and Table F4 contains the pH measurements for all the Pheroid® formulations used in the preservative efficacy test. At stability interval 48 hours the formulations without added organisms were measured. After 28 days the measurements for each formula with the five test organisms under investigation were measured and compared to the initial values obtained.

The parabens are effective over a pH range of 4 to 8. As the pH increases above 8, the parabens become less effective due to dissociation of the molecule. At a pH of 8.5, 50% of the compound is dissociated and considerable loss of antimicrobial activity occurs. At pH 7, the parabens are 60 to 65% effective (Haag & Loncrini, 1984: 70).

All the formulas prepared showed an initial pH value in excess of 6, measured at time 48 hours. After 28 days had elapsed and with the addition of the cultures, the pH values measured lowered throughout the range of formulations prepared. All the values measured were still above the required pH value of 4 to be effective; they varied between 4.444 and 5.614 at 28 days. Possible influences on the pH may be the duration of the storage and the addition of cultures to the formulas.

Preservative efficacy test

Annexure G presents the PET results obtained for each individual formula over the 28 day test period. The counting of bacteria was done according to the spread plate counting method in section 3.8.6, with the interpretation of the data done according to the criteria given in section 3.8.7.

For each formula prepared, a control of peptone water without added preservatives, were used for the bacterial cultures, with candida and Tween® 80 water used for the aspergillus strains. PG had the added advantage that it potentiated the antimicrobial activity of the parabens in the presence of non-ionic surfactants and prevented the interaction between ethylparaben and polysorbate 80 or Tween® 80. According to Haag and Loncrini (1984:65), in studies performed using parabens as choice of

preservative, parabens are not particularly effective against *Pseudomonas aeruginosa*.

The Pheroid® formula tested, without any preservatives added to the formula, met the criteria for oral formulations tested according to the PET. At time 14 days, no visible growth were detected and for the remainder of the 28 days the Pheroid® formula proved fatal to all five test organisms. Future tests will try to determine the efficacy of the Pheroid® components to self-preserve the system. The addition of PG (10% v/v) to the Pheroid® formula, still without added preservatives, also passed the criteria of the PET.

The formula with added Methylhydroxybenzoate (MHB 0.1% w/v) preserved the formula against all five cultures, although the formula with an extra 10% v/v PG could not meet the criteria of preservation for the *Staphyloccocus aureus* culture. The Ethylhydroxybenzoate (EHB 0.15% w/v) formula had an increase in the *Staphyloccocus aureus* culture at 28 days as well as the *Candida albicans* cultures. When PG (10% v/v) were added to the EHB formula, the formula met the criteria for the PET for all cultures. Propylhydroxybenzoate (PHB 0.015% w/v) could not protect the formula against the growth of *Candida albicans*. The addition of PG (10% v/v) had no further effect on the efficacy of the formula containing PHB and the PET failed. Butylhydroxybenzoate (0.03% w/v) with and without added PG (10% v/v) proved fatal for all the test formulas.

Nipasept® consists of a combination of three paraben preservatives namely methylhydroxybenzoate, ethylhydroxybenzoate and propylhydroxybenzoate. This formula with Nipasept® (0.175% w/v) could not pass the criteria for *Candida albicans* as an increase in growth was counted at day 28. With added PG (10% v/v) the PET passed for all five organisms.

The second combination product used was Nipastat® at a concentration of 0.175% w/v. The components of Nipastat® are MHB, EHB, PHB, BHB and isobutylparaben. Both formulas passed the PET with no detection of any cultures from day 14 to day 28.

Visible detection

All the formulas prepared for the preservative efficacy test were stored in a cupboard and reopened at the predetermined test intervals to distinguish the preservative efficacy. At the end of the 28-day period all the formulas had discoloured to yellow due to possible oxidation of the fatty acids. For the accelerated stability test the end formula will be packed into single foil sachets to prevent the oxidation process.

3.9 PRESERVATIVE EFFICACY TEST PROCEDURE FOR PHEROID® WITHOUT ADDED PRESERVATIVE

When the positive results obtained with the Pheroid® formula displayed self-preservation, it was decided to repeat the experiment once more. The manufacturing sheet for the repeat of the preservative efficacy test is attached in Addendum H. Similar results were obtained as before (see section 3.8.8.3). No growth was detected for any of the five organisms tested (see preservative efficacy test results in Annexure H).

Table H1, in Annexure H, contains the summary of the particle size values and pH measurements done after 48 hours and 28 days with the five organisms added to the formula. The D (0.9) value for the formula after 48 hours measured 0.266 µm. After the addition of the bacterial cultures, the *Pseudomonas aeruginosa* sample measured a particle size of 291.917 µm, which might be due to the detection of big colonies in the formula sample. *Staphylococus aureus* and *Eschericia coli* measured 0.265 µm and 0.290 µm respectively, the *Candida albicans* culture measured a particle size value of 348.133 µm and the *Aspergillus brasilliensis* had a particle size of 266.658 µm.

The pH value for the initial sample measured at 48 hours was 6.281. After 28 days the values lowered for all five samples with added organisms to between 5.423 and 4.843.

3.9.1 EXPERIMENTAL OUTLAY

To determine the contributing factors of the self-preservation ability of the Pheroid®, the basic Pheroid® formula (see section 2.7.2.1) was altered to six formulas each without a basic excipient of the Pheroid®. The manufacturing sheets for the six formulas can be seen in Annexure I.

The formulas tested were lettered A to F and varied with the omission of the following excipients.

Test formula A: The basic Pheroid® formula, as control for the PET.

Test formula B: The basic Pheroid® formula with normal distilled water used in place of N_2O saturated water.

Test formula C: The basic Pheroid® formula without the emulsifier, Cremophor® EL.

Test formula D: The basic Pheroid® formula without the anti-oxidant, dl-α-tocopherol.

Test formula E: The basic Pheroid® formula without the anti-oxidants, BHA and BHT.

Test formula F: The basic Pheroid® formula without the anti-oxidant, TBHQ.

3.9.2 RESULTS

The influence of the exclusion of each excipient was tested over a 28-day period. The tests used to examine the stability of each formulation were pH measurements, particle size, preservative efficacy test, CLSM as well as visual assessment. The stability intervals for each test were noted after 48 hours and 28 days.

3.9.2.1 Particle size

Table I1 in Annexure I summarises the particle size values measured at 48 hours and 28 days. The only formulation exhibiting a pronounced increase in particle size, or droplet size, is explainable by the exclusion of the emulsifier, Cremophor® EL. Sample C measured D (0.9) values of 91.886 µm at 48 hours. After addition of the five test organisms, the values measured after 28 days were between 120.739 µm and 177.156 µm (see Figure 3.11). Another factor taken into consideration was the influence of storage of the single samples without added organisms at a temperature of 4ºC. The particle sizes were, on average, not influenced by the extended storage period of 28 days.

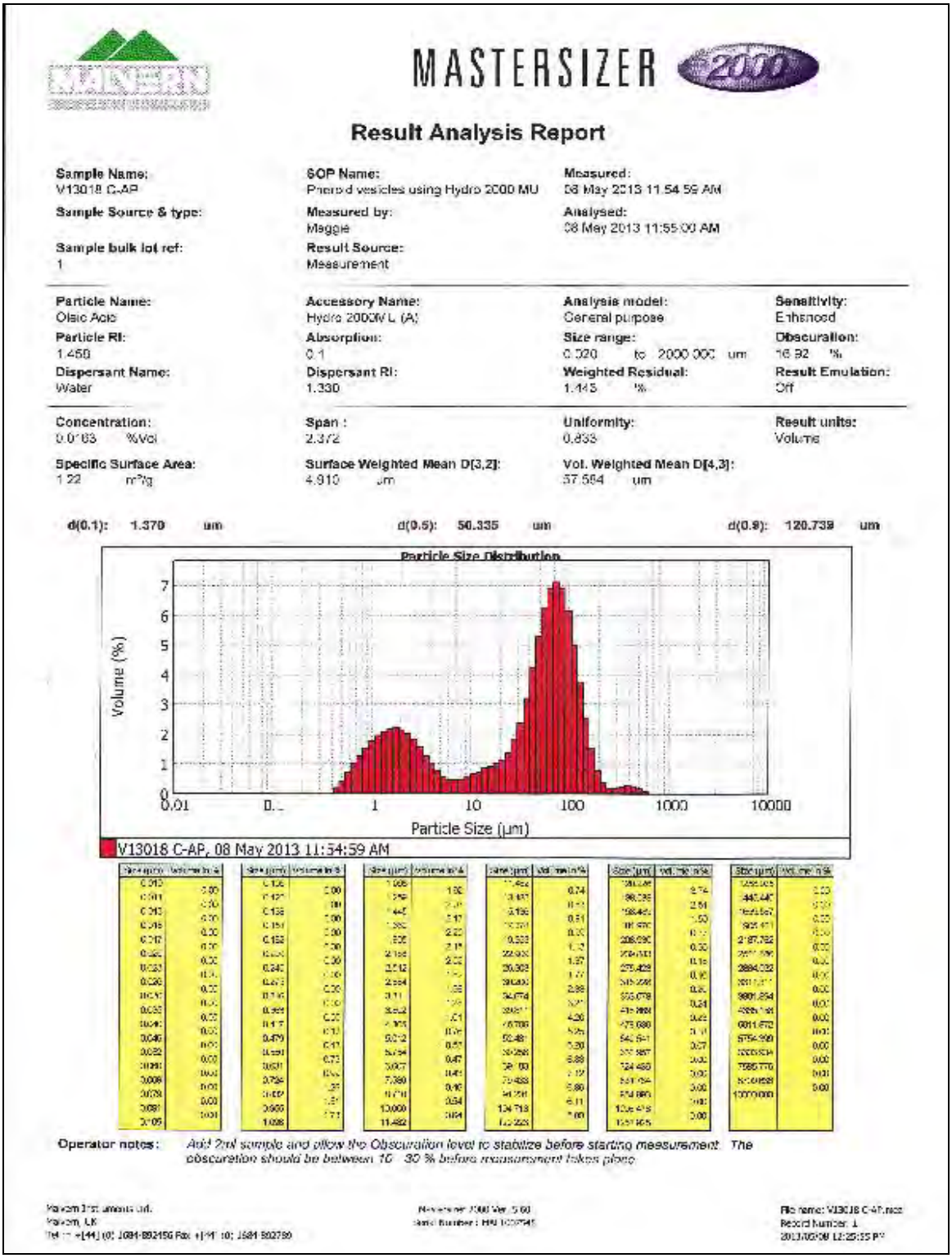

Figure 3.11: Mastersizer result analysis report for Formula C (with added *Aspergillus brasilliensis*) as an example of the influence of the removal of the emulsifier component on the particle size distribution. D (0.9) value obtained shows that 90% of the particles were found around ± 120 µm.

3.9.2.2 pH

Table I2 in Annexure I summarises the pH values measured at 48 hours and 28 days. At time 48 hours, the pH values of the six formulas prepared varied between pH values of 6.535 and 7.203. With the addition of the organisms and after 28 days the pH values of the six formulas varied between pH values of 4.779 and 6.490.

3.9.2.3 Preservative efficacy test

Annexure I contains the preservative efficacy results of all six formulas. All the formulas tested passed the PET for all five test organisms, with the exception of Formula F (basic Pheroid® formula without the antioxidant, TBHQ). For this formula the *Aspergillus brasilliensis* culture did not show a 1.0 log reduction as determined in the criteria of the BP, although Envirocare Laboratories makes use of the USP (see Annexure I) which determines moulds for category 3 products should show no increase from the initial calculated count at seven, 14 and 28 days. In this instance, Formula F would inhibit the growth of the *Aspergillus brasilliensis* but not show fungicidal effects as with the other formulas investigated. Section 2.5.3 describes the advantages of TBHQ as an anti-oxidant for oily preparations. It can be directly related that TBHQ in the Pheroid® formula had the advantage of preserving the formula against the mould *Aspergillus brasilliensis.* The outcome of this experiment confirms data collected in similar tests done in section 3.6.1 by Grobler and Wilken and Grobler. The results obtained for sample B (basic Pheroid® formula without the nitrous oxide saturated water but using normal distilled water instead) proved that nitrous oxide alone does not have an effect on the preservative efficacy of the Pheroid® but a combination effect of the fatty acids and other ingredients may determine the outcome of self-preservation.

3.9.2.4 Confocal laser scanning microscopy

See Figure 3.12 of CLSM photos taken of all six formulas with individual components removed compared to sample A (the basic Pheroid® formula). The photo taken of sample C shows the oil patches in the formulation responsible for the oil layer on top of the formula and the cause of the increased particle size values. Formulas A, B, D, and E did not show any visual differences with the exclusion of their individual components. Sample F confirms the particle size measurements obtained for this sample which showed an increase in the average particle size measured at 48 hours of ±3.0 µm compared to the ± 0.3 µm results obtained for formulas A, B, D and E.

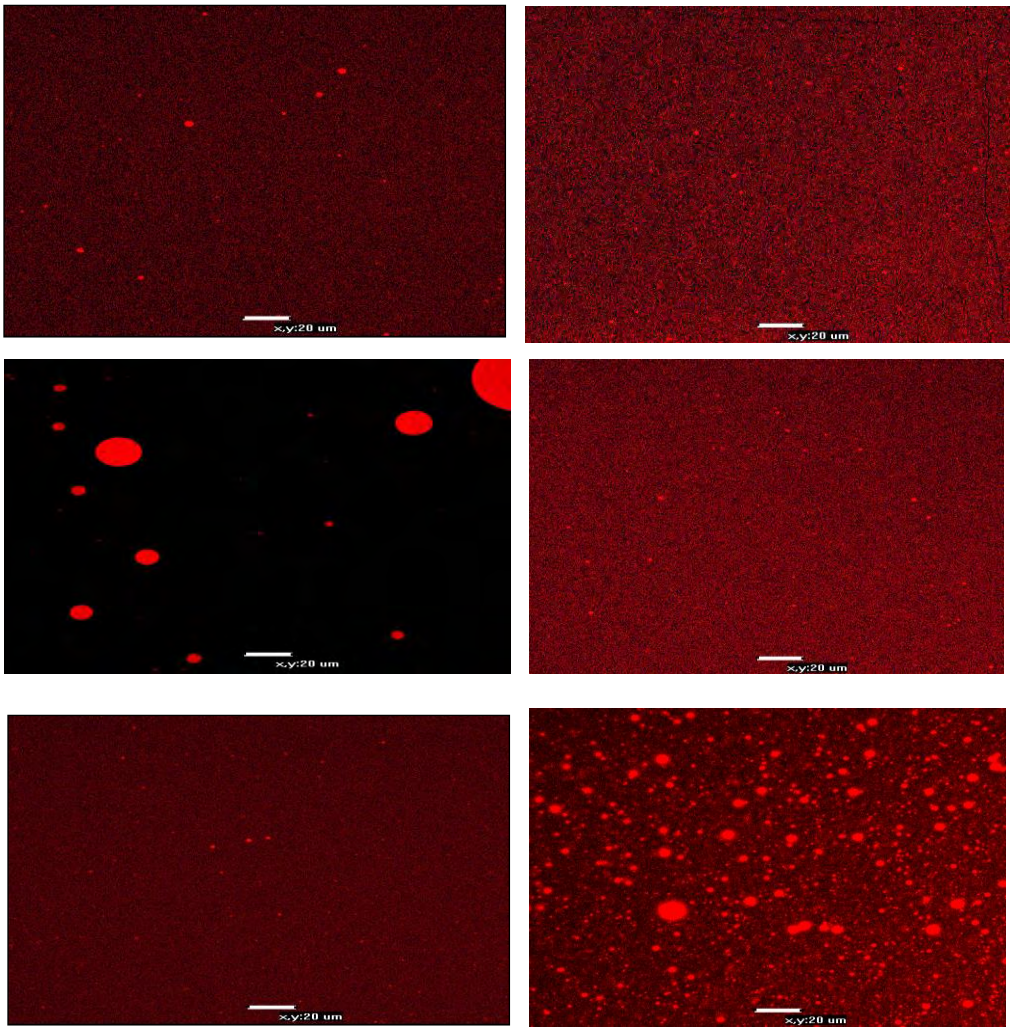

Figure 3.12: CLSM photos taken of sample A (top left), sample B (top right), sample C (middle left), sample D (middle right), sample E (bottom left) and sample F (bottom right) at 28 days.

3.9.2.5 Visible detection

All the formulas initially appeared to be uniform white emulsions with a light pink colouring with the exception of formula C, which had divided into layers of oil in water. A top layer of yellow oil could be seen for the formula without the added emulsifier, Cremophor® EL. After 28 days all the formulas discoloured to between light yellow and dark yellow. The exclusion of the anti-oxidants (BHA, BHT and TBHQ), individually proved that a combination of anti-oxidants is necessary to preserve the formula against oxidation. The formulas were kept at normal room temperature, which contributed to the discolouring, compared to the normal recommended storage temperature of 4°C.

3.9.3 CONCLUSION

It is not common practice to include antimicrobial preservatives in anhydrous ointments because microorganisms, while they may survive, rarely proliferate in such systems. However, in polyphasic ointments that contain water, in aqueous gels and creams (particularly emulsions of the oil-in-water type) antimicrobial systems should be included to prevent both spoilage and the growth of pathogenic organisms (Lund, 1994:151).

A preservative should ideally be non-toxic and non-allergenic, have a bactericidal rather than a bacteriostatic action and be active against a wide range of microorganisms. As well as being inexpensive and potent it should be stable under a wide range of storage conditions, be free from unpleasant odour or strong colour, and be unaffected by other formulation ingredients and packaging materials (Lund, 1994:151).

The hydroxybenzoate esters (methyl, ethyl, propyl and butyl) are stable, odourless and virtually nontoxic. However, they have a low aqueous solubility and show weak activity against Gram-negative bacteria; they have also been associated with allergic reactions. PG, in sufficient concentration, can also function as a preservative (Lund, 1994:151).

Much consideration was given to determine a formula safe to administer to patients. The formula that proved to be efficient was Nipastat® 0.175% w/v, along with PG10% w/v. This product did not increase in mean particle size; pH value was still within the desired pH range of 4 to 8 and passed the PET with ease. The added advantage of a combination product is the lowered individual concentrations necessary of each component in the product which has fewer toxicity and side effects. The combination product improved activity towards potential contaminants added to the formula either during manufacturing or by patient use and incorrect storage methods followed. In Chapter 4 the preservative Nipastat® was tested over a three month period in an accelerated stability test under various stress conditions.